Microbial Diversity in the Human Mouth: Dominant Genera and their Interactions.

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Abstract.

Investigations seeking definition of the microbial diversity present in the human oral cavity have been underway for many years. These investigations seek to define the normal microflora of the oral cavity, as well as the stability of the microflora. Predominantly, these kinds of studies have focused on the differences in microbial carriage between healthy and diseased oral cavities and have identified several species which may be causative and promote oral diseases such as periodontitis and halitosis. Less is known about how co-habitants of a healthy oral cavity interact and how those interactions are mediated and controlled. The genes that may be involved in microbial interactions and their regulation and roles within response pathways in oral microbes is an interesting area of research to pursue, as it could give valuable insight into the changing genetic and potentially physiological state of oral microbes.

An evaluation of oral microbial diversity performed during this study by Restriction Fragment Length Polymorphism (RFLP) and Fatty Acid Methyl Ester (FAME) profiling analyses carried out on whole mouth rinse samples identified many bacterial genera from six phyla. These genera included *Streptococcus*, *Gemella*, *Veillonella*, *Eubacterium*, *Porphyromonas*, *Neisseria*, *Rothia* and *Prevotella*. The three prevalent genera, as defined by the number of times observed from mouth rinse samples, were *Streptococcus*, *Neisseria* and *Veillonella*.

Solid-phase co-culture experiments using isolates of the three prevalent genera revealed a strong interaction between *Streptococcus* and *Neisseria*, with *Streptococcus* exerting a negative effect on Neisserial growth. Liquid phase co-culture experiments between these two genera confirmed initial observations and it was hypothesised that the interaction was mediated by toxic hydrogen peroxide produced by *Streptococcus*. Further experiments showed that *Neisseria* were protected from

Streptococcal killing upon addition of exogenous catalase, confirming that the interaction was mediated by hydrogen peroxide.

The roles of the key genes of the oxidative stress response of *Neisseria* were investigated by assessing the interactions between *Streptococcus pneumoniae* and several mutant *Neisseria meningitidis*, defective in one or more genes of the oxidate stress response. These experiments resulted in observations differing to those previously published, particularly in relation to the *prx* gene (encoding the peroxidase peroxiredoxin), which lead to the proposal of multiple pathways of stress response existing in *N. meningitidis*. It was observed that *N. meningitidis* survival was increased in the absence of the *bcp* gene (encoding the peroxidase bacterioferritin co-migratory protein) and it was hypothesised that this was due to an up-regulation of other peroxidases in this mutant. Real-Time PCR (RT-PCR) experiments showed increased expression of *prx* in a *bcp*-deficient *N. meningitidis* mutant in the presence of hydrogen peroxide, but this up-regulation was only marginal in the un-stressed mutant strain.

Furthermore, it was shown that in the presence of high-levels of hydrogen peroxide (1 mM), *N. meningitidis* and the various mutant strains examined, behaved as previously observed. This gives rise to the notion that the hydrogen peroxide levels produced in co-culture with *S. pneumoniae* are not high enough to induce the typical oxidative stress response in *N. meningitidis*, indicating that another pathway(s) must exist in order to deal with low / intermediate-level hydrogen peroxide / organic peroxides experienced during co-colonisation of the oral cavity.

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1. General Introduction.

1.1 Establishing microbial diversity within the oral cavity – molecular analyses.

Establishing the diversity within the oral cavity is an area of research that has been pursued for many years. At first, investigations into identifying microbes were carried out using culture-dependent techniques (Querido et al., 1976), but these severely underestimate the diversity of the microbial community (Amann et al., 1995, Hugenholtz et al., 1998). It is now known that less than 1% of the microbes found in many natural environments are culturable (Hugenholtz et al., 1996), therefore a huge percentage of the microbial flora is being ignored. Some microbes cannot be cultured, as an appropriate growth medium has not been developed. The bacteria may not be able to grow well enough under laboratory conditions, making experiments impossible (Paster et al., 2006). There is also a mixture of aerobic and anaerobic microbes in the oral cavity, and culture of anaerobes in the laboratory could be difficult, preventing identification of them (Paster et al., 2006). For these reasons, a culture-independent method of study is needed, to provide a more comprehensive view of the microbial flora within the human mouth.

With advances in molecular biology, these culture-independent techniques have been developed, making the assessment of microbial diversity much faster and more effective. Results can be achieved via the use of restriction fragment length polymorphism (RFLP) analysis. This technique relies on DNA extraction from bacterial communities, followed by amplification of the 16S ribosomal RNA (16S rRNA) genes, which are known markers of relatedness between species (von Witzengerode et al., 1997). These genes can be easily amplified using universal bacterial primers (Hugenholtz et al., 1998, Sakamoto et al., 2003, de Lillo et al.,

2006). The samples used for this analysis come from a wide range of environments, including subgingival plaque (Paster et al., 2001), other oral sites (Paster et al., 2006), the pharynx (Brook, 2003) and female reproductive organs (Zhou et al., 2004). It is also possible to gain knowledge of the archaeal members of and environment by using archaeal-specific primers (Takai and Horikoshi, 1999, Reysenbach et al., 2000). Once amplified, the 16S rRNA gene can be isolated and purified, before being cloned into a vector and subjected to restriction digestion, producing RFLP banding patterns. Each different banding pattern is known as a phylotype and each different phylotype can be sequenced, to identify the species from which the 16S rRNA gene was amplified.

A further method of molecular analysis, which is much less time-consuming, is terminal restriction fragment length polymorphism (T-RFLP). This method uses fluorescently labelled primers to amplify the 16S rRNA genes. The products are digested and subjected to fragment analysis by capillary electrophoresis. This method has been used successfully to investigate microbial diversity in a variety of environments (Sakamoto et al., 2003, Clement et al., 1998, Dunbar et al., 2000, Hiraishi et al., 2000).

More recently, due to continuously improving technologies, high-throughput sequencing technologies, such as 454 sequencing have dramatically increased the volume of DNA sequencing possible, leading to vast amounts of sequence data being obtained quickly (Rothberg and Leamon, 2008).

Preparation for 454 sequencing includes shearing genomic DNA into fragments of a few hundred bp, which are then linked to microbeads in a 1:1 ratio (Wicker et al., 2006). The microbeads are captured in droplets of an emulsion, which serve as PCR microreactors for template amplification (Wicker et al., 2006). These are then distributed into a fibre-optic slide (known as a PicoTiterPlate™), where the four DNA nucleotides are added in turns (Wicker et al., 2006). Integration of a nucleotide into a DNA strand in one of the wells is translated into a light signal by the enzyme luciferase

(Wicker et al., 2006). Raw DNA sequences are assembled into sequence contigs which, in the finishing phase, are connected properly to the final sequence (Wicker et al., 2006).

Unfortunately, funding was not available for use of such high-throughput sequencing for this project.

1.2 Pitfalls of molecular methodologies.

Although these molecular methods provide a more detailed view of microbial diversity, it is important to realise that they can become biased, affecting the results obtained. Before amplifying the 16S rRNA gene, cell lysis has to be performed to release the DNA. If lysis is inefficient or incomplete, some DNA remains in the cell and is excluded from the results. On the other hand, if lysis conditions are too stringent the DNA may become fragmented, leading to incorrect PCR products being obtained (von Witzengerode et al., 1997). Large amounts of DNA can be lost during nucleic acid purification steps, leading to a reduced amount of genetic material available for analysis (von Witzengerode et al., 1997).

It is also possible that contaminants, from reagent tubes or poor sample handling techniques, could affect the PCR reaction, leading to a loss of, or formation of incorrect products due to preferential primer binding to the contaminating DNA (von Witzengerode et al., 1997). Finally, there may be differential amplification during the PCR reaction, due to differing efficiencies in both primer annealing and extension steps and experiments become particularly prone to this when relying on universal bacterial primers (von Witzengerode et al., 1997). This differential amplification can also occur because the sample analysed contains a complex mixture of homologous genes, with a mixture of sequences that may promote

preferential primer binding (von Witzengerode et al., 2007, Suzuki and Giovannoni, 1996).

1.3 Fatty acid methyl ester (FAME) profiling.

One further culture-independent method used in measuring microbial diversity is fatty acid methyl ester profiling (FAME profiling). This is a rapid, cost effective method and has been widely regarded as an accurate way to identify bacteria from pure cultures (Stead, 1995) and more recently, this method has been used to extract FAMEs from mixed samples such as soil, compost and fungus, allowing conclusions to be drawn on the diversity present within such communities (Ritchie et al., 2000, Carpenter-Boggs et al., 1998, Graham et al., 1995). The type and percentage of individual fatty acids present in bacteria not only varies from genus to genus, and species to species, but in some cases it is also unique at biovar or pathovar level (Stead, 1995). FAME profiling provides a powerful tool which allows for identification of many, but not all bacteria. The methodology uses the Sherlock® Microbial Identification System (MIDI) which is used worldwide in clinical and environmental laboratories to identify over 1500 aerobic and anaerobic bacterial species (http://www.midi-inc.com).

In order to analyse and identify the bacteria under investigation it is necessary to obtain sufficient weight of cells (approximately 40 mg). To meet this requirement, cells are cultured under strict conditions, according to the appropriate MIDI protocol, until cultures are pure and contain sufficient material for analysis. Cells can then be harvested for analysis. Once harvested, the fatty acids can be extracted, converted to fatty acid methyl esters (FAMEs), and analysed. Fatty acid profiling relies on separating the extracted FAMEs according to their size and conformation.

This procedure uses a Hewlett Packard HP6890 series Gas Chromatograph. The Hewlett Packard HP6890 series GC is controlled by the HP Chemstation software which interfaces with Midi Sherlock software (MIDI Inc., USA). This software enables comparison with a commercially available library (TSBA6). Once the samples are analysed, the resulting profiles are compared to the libraries and an analysis report is automatically generated.

Once the sample has been analysed a profile is produced which identifies and quantifies the fatty acids. A comparison is made to the library entries and a profile is produced complete with a similarity index, and histoplots showing the values of the sample compared to the nearest library entries (Sherlock Microbial Identification System Operating Manual, Version 6.0, 2005). Individual profiles are produced for each sample analysed, providing much data about the species diversity present in a number of samples at any one time.

1.4 Species diversity within the oral cavity in health and disease.

Over 700 bacterial species have been identified from studies of the oral cavity (Paster et al., 2006), with many studies focusing on the diversity in the oral cavity during disease (Paster et al., 2001, Sakamoto et al., 2005). Studies that have examined microbial diversity in several sites of the oral cavity have identified many bacterial taxa from six phyla as being components of healthy oral cavities. These comprise of Firmicutes, including *Streptococcus*, *Gemella*, *Eubacterium*, *Selenomonas* and *Veillonella* species (Paster et al., 2001, Aas et al., 2005, Kazor et al., 2003), Actinobacteria, including *Actinomyces*, *Atopobium* and *Rothia* species (Aas et al., 2005, Kazor et al., 2003), Proteobacteria, including *Neisseria*, *Eikenella* and *Campylobacter* species (Paster et al., 2001, Aas et al., 2005), Bacteroidetes, including *Porphyromonas*, *Prevotella* and *Capnocytophaga* species (Paster et al., 2001, Aas et al., 2005, Kilian et al., 2006), Fusobacteria, including *Fusobacterium* and *Leptotrichia* species (paster et al., 2001, Aas et al., 2005) and members of the TM7 phylum (Aas et al., 2005, Kazor et al., 2003, Brinig et al., 2003). This phylum has no culturable members, but bacteria belonging to this phylum are seen in both healthy oral cavities and in oral diseases such as halitosis (bad breath) and mild periodontitis (wastage of the gums) (Aas et al., 2005, Kazor et al., 2003).

The main studies focused on disease of the oral cavity have focused on periodontal disease, that is, disease of the gums that is initiated or accelerated by subsets of microbes that colonise on, or just below the gingival margin (Haffajee et al., 1998). Studies have been underway for over one hundred years to investigate the microbial causes of such disease (Haffajee et al., 1998), with significant progress made in the last fifteen years, following the identification of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythia* as pathogenic contributors to periodontal disease (Haffajee et al., 1998, Teles et al., 2006). Additional pathogens that are also thought to

contribute to the acceleration of periodontal disease are *Treponema denticola* (a member of the Spirochaete phylum) (Sakamoto et al., 2005. Paster et al., 2001), and *Prevotella intermedia* (Sakamoto et al., 2005, Kilian et al., 2006). It is thought that the Spirochetes are the predominant proliferating microbes associated with sites of periodontal disease (Sakamoto et al., 2005).

Another oral disease of importance is halitosis (bad breath), which is a complaint of approximately one third of the population and can also be positively correlated with patients suffering from periodontal disease (Kazor et al., 2003). This condition can be caused by a variety of sources, such as the gastrointestinal tract and the lungs, but most commonly arises from the oral cavity (Kazor et al., 2003). The production of malodorous compounds occurs on various oral surfaces and in periodontal pockets, but occurs most often on the tongue dorsum, with the malodour produced from bacterial metabolic processes and fermentation of oral proteins (Kazor et al., 2003).

The most obvious and offensive malodorous compounds are termed volatile sulfur compounds (VSCs) and include hydrogen sulfide, methyl mercaptan, and dimethyl sulphide, which together, account for around 90 % of all VSCs (Kazor et al., 2003). Other malodorous compounds include short-chain organic acids such as valeric acid, butyric acid, putrescine, and skatole (Kazor et al., 2003). Species that produce such malodorous compounds include *Treponema denticola*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis*, *Porphyromonas endodontalis*, and *Eubacterium* species (Persson et al., 1989 and 1990, Kazor et al., 2003).

The species most associated with health in the study of halitosis by Kazor et al. were *Streptococcus salivarius*, *Rothia mucilaginosa* and an uncharacterized species of *Eubacterium* (strain FTB41), whereas the prevalent microbes present on the tongue dorsa of patients suffering with halitosis was different, with *Atopobium parvulum*, a phylotype of *Dialister*,

Eubacterium sulci and a phylotype of the uncultivated phylum TM7 all being identified (Kazor et al., 2003).

1.5 Bacterial biofilms and quorum sensing – interactions during plaque formation.

Biofilms can be described as a matrix-enclosed aggregation of bacteria, with patches of cell aggregates being found throughout a polysaccharide matrix (Stoodley et al., 2004, Davey and O'toole, 2000). The matrix varies in density, thus creating open areas of the biofilm, where water channels can be found (Davey and O'toole, 2000). Forming a biofilm offers protection to the bacteria from environmental stresses such as UV exposure, metal toxicity, phagocytosis and antibiotics (Stoodley et al., 2004).

Biofilms are an important element of the oral cavity as they are present as plaque upon tooth surfaces. Furthermore, the carrying bacterial species residing within the plaque biofilm must interact in order to create and maintain the biofilm. *Streptococcus* and *Actinomyces* are the major initial colonisers of a plaque biofilm (Kolenbrander, 2000) and they help to establish the early biofilm community.

In order for bacterial biofilms to mature, interactions must occur between the bacteria comprising the biofilm. The most notable of these interactions is coaggregation, which can be defined as the recognition and adhesion between genetically distinct bacteria (Davey and O'toole, 2000). Studies investigating bacterial coaggregation have shown that the pioneer coloniser *Streptococcus gordonii* can coaggregate with *Fusobacterium nucleatum*, but it cannot coaggregate with the late biofilm coloniser *Actinobacillus actinomycetemcomitans* (Kolenbrander, 2000, Davey and

O'toole, 2000). *F. nucleatum* can coaggregate with both species, hence, is known to act as a bridge between early and late plaque biofilm colonisers. There is evidence to show that coaggregation occurs via species specific interactions between bacteria from the same location within the oral cavity (Davey and O'toole, 2000). *Fusobacteria* also play a role in bacterial plaque biofilms by acting as physiological bridges to promote anaerobic microenvironments (Kolenbrander, 2000). The promotion of such environments and interactions between anaerobes protects the anaerobic bacteria residing within the biofilm (Kolenbrander, 2000).

There are further interactions which take place within a bacterial biofilm, such as substrate exchange and removal / redistribution of metabolic products (Davey and O'toole, 2000). Biofilms are also important for the development of syntrophic relationships between bacteria, where two metabolically distinct species rely on each other for substrate utilisation and energy production (Davey and O'toole, 2000). Short-chain fatty acids produced by oral bacteria are thought to be an essential carbon source for certain oral bacteria (Hojo et al., 2009). It has also been suggested that a symbiotic association between *Streptococcus* and *Veillonella* species may exist, due to lactic acid production by *Streptococcus* (Hojo et al., 2009). However, in spite of the positive interactions that occur within bacterial plaque biofilms, heavy competition between biofilm species will occur, due to restricted nutrient levels and competition for space within the biofilm (Hojo et al., 2009). Bacteriocins are a likely bacterial weapon that can be used to overcome such competitive strains. It is thought that mutacins may allow *Streptococcus mutans* to invade the dental biofilm community and to colonize there, as the mutacin activity of this species is likely related to its successful establishment in the dental biofilm (Hojo et al., 2009). The interaction thought to be responsible for gene transfer in bacterial biofilms is conjugation. This interaction has been studied in dental plaque biofilms, where it has been shown that *Streptococcus* species can take up a transposon responsible for tetracycline, originating

from *Bacillus subtilis*, whilst residing in a fixed-depth biofilm (Roberts et al., 1999).

The interactions discussed so far in this section are all governed by responses to the environment and subsequent changes in gene expression. The process by which regulation of gene expression and subsequent behaviour of bacteria changes as a result of bacterial population density is known as quorum sensing (Miller and Bassler, 2001, Spoering and Gilmore, 2006). Quorum sensing bacteria produce chemical signals known as autoinducers, which increase in concentration as the population density increases (Miller and Bassler, 2001). The main autoinducer communicator molecules are acylated homoserine lactones (AHLs), used by Gram negative bacteria and oligo-peptides, used by Gram positive bacteria (Miller and Bassler, 2001).

AHLs are small molecules produced by the LuxI family of proteins and are produced at a constant rate by the bacterial cell, accumulating as the cell density increases (Spoering and Gilmore, 2006). AHLs affect transcription by diffusing into the cytoplasm and binding to a LuxR homologue specific to each AHL (Spoering and Gilmore, 2006). The LuxR family of DNA-binding proteins binds to a conserved promoter sequence and can positively or negatively affect transcription and the architecture of the biofilm (Spoering and Gilmore, 2006).

One example of the peptide autoinducers involved in quorum sensing is the Streptococcal competence signalling peptide (CSP). In the case of *Streptococcus pneumoniae*, when the CSP reaches a threshold of 1–10 ng/ml in the medium, a subpopulation of the bacteria lyse, and the released DNA is taken up by the remaining population (Spoering and Gilmore, 2006). In addition to its role in DNA uptake, the CSP system has been shown to contribute to biofilm formation in several species of *Streptococcus* (Spoering and Gilmore, 2006). As discussed previously in

this section, it is known that *Streptococcus* species are pioneer colonisers of oral plaque biofilms, so it is reasonable to assume that quorum sensing plays a large role in this process, especially when concerning those Streptococcal populations inhabiting the same, or very close oral locations.

The detection of a threshold concentration of these autoinducers by other bacterial species leads to an alteration in gene expression and physiological processes such as symbiosis, conjugation and motility (Miller and Bassler, 2001). It is also known that quorum sensing induces biofilm formation (Miller and Bassler, 2001). This indicates that quorum is likely to be an important bacterial interaction occurring within the oral microbial flora, especially at the initial stages of plaque formation and maturation.

1.6 Antimicrobial activity of the oral cavity – Defence against pathogens.

The oral cavity is the first line of defence against ingested pathogens. Pathogens such as *E. coli* and *Salmonella* can enter the mouth if present on food but rarely cause infection. This is due to several defence mechanisms preventing pathogens from entering the body. Biting, chewing, salivary flow and tongue movements are all mechanical ways that microbes can get dislodged from surfaces within the mouth and washed down to the stomach, where they will be killed by the strong acidic conditions found there.

There is an innate immune system within the oral cavity, consisting of many proteins, including microbial proteins such as lysozymes, lectins, lactoferrins and transferrins (Zasloff, 2002). Alongside these proteins, antimicrobial peptides have been discovered (Zasloff 2002, Boman, 1995), which are produced by a variety of oral tissues. In humans, the major classes of these peptides are defensins (Lehrer and Ganz, 2002) and cathelicidins (Gennaro and Zanetti, 2000) and they exhibit a broad spectrum of activity against bacteria, viruses and fungi (Zasloff, 2002). Both classes of peptides act to protect the tongue and gingiva (gums) and are generally present in high levels after injury (Zasloff, 2002). They are also thought to provide a link between the innate and adaptive immune system, as when antimicrobial peptides are expressed, immune cells are attracted to the site of expression (Zasloff, 2002).

Toxic compounds are also produced in the oral cavity to defend against pathogens. One of these compounds is nitric oxide (NO). This is produced by host tissues via the inducible enzyme, nitric oxide synthase, isoform 2 (NOS2) (Fang, 1997). This is stimulated to form NO from arginine in the mouth, by pro-inflammatory cytokines, microbial lipopolysaccharide (LPS) and microbial lipoteichoic acid (Fang, 1997). Increased NO levels are seen in response to trauma and infection (Fang,

1997, Ochoa et al., 1991) and increases in NOS2 expression and NO levels have been seen in the human bacterial infection tuberculosis (Nicholson et al., 1996). NO-related antimicrobial activity has been seen against bacteria, viruses and fungi (Fang, 1997, Degroote and Fang, 1995), demonstrating a wide range of defence.

Nitric oxide produced as a defence in the mouth has been shown to decrease the growth of the oral pathogens *Fusobacterium nucleatum*, *Eikenella corrodens* and *Porphyromonas gingivalis* (Allaker et al., 2001), as well as the pathogens *Streptococcus mutans*, *Lactobacillus casei* and *Actinomyces naeslundii* (Mendez et al., 1999). The nitrate present in saliva encourages nitrate-reducing bacteria e.g. *Veillonella*, *Actinomyces*, *Rothia* and *Staphylococcus epidermidis* to reside in the mouth (Doel et al., 2005). Evaluation of nitrate reduction in the mouth identified *Veillonella* to be making the largest contribution to nitrate reduction (Doel et al., 2005).

1.7 Interactions between microbes in the oral cavity.

The oral cavity contains complex, multi-species microbial communities, with the total number of species colonising the mouth being greater than 700 (Aas et al., 2005, Paster et al., 2006). Therefore, it is not unreasonable to presume there are many interactions occurring between members of this community.

There are several factors which can determine the interactions taking place within the oral cavity and indeed, some interactions may rely on a combination of more than one of these factors.

The first important factor in determining the bacterial composition of a biofilm and microbial community and interactions therein, is the availability of nutrients, coupled with the ability of the bacteria to metabolise these nutrients effectively (Kuramitsu et al., 2007). Nutrients are available from the intake of food during eating meals, saliva, production by other organisms and dental plaque (Kuramitsu et al., 2007, Bowden, 1997). The ability to metabolise sucrose may be a competitive advantage, as there are high levels of sucrose in many human diets (Kuramitsu et al., 2007). A competitive advantage may also be sought for those bacteria that are able to survive well in the presence of acid that is produced as a metabolic by-product by some bacterial species (Dorana et al., 2004, Greiner, 1996). The proteins present in the oral cavity are also a useful source of nutrients for bacteria (Kuramitsu et al., 2007). Having proteolytic properties will therefore present a great competitive advantage to a species, allowing growth under limiting nutrient conditions (Kuramitsu et al., 2007).

Some pathogenic bacteria are known to have formed symbiotic relationships in order to interact and co-inhabit niches within the mouth. This is certainly the case for *Porphyromonas gingivalis*, one of the major causes of periodontal disease, which is known to coexist with several

pathogenic species (Moore et al., 1982, Dzink et al., 1988). It has also been shown that *P. gingivalis* can use the metabolic by-products of its co-habitants in order to promote growth and that in turn, its own metabolic by-products can be utilised by the species it co-habits with, resulting in a symbiotic existence (Greiner, 1992). These interactions may also enhance overall pathogenic growth rate and virulence of disease (Yoneda et al., 2001, Kuramitsu et al., 2007).

Mutualistic interactions are also known to occur between organisms in the mouth and may be beneficial to those species which are poor single-species colonisers, but are able to colonise and form biofilms in the oral cavity when interacting via their metabolic systems (Palmer et al., 2001, Kuramitsu et al., 2007). Furthermore, metabolic products of one organism may promote or facilitate the growth of other organisms co-inhabiting a niche of the oral cavity, during cooperative metabolic interactions (Kuramitsu et al., 2007).

Bacteria are also able to generate products which exert specific effects on other bacteria, with a good example of this being the expression of toxic bacteriocins, also known as mutacins, by some species (Chikindas et al., 1995, Qi et al., 2001, Kuramitsu et al., 2007), which kill, or inhibit the growth of related / co-colonised species. Many oral bacteria have been shown to use bacteriocin-like compounds to compete with other species and inhibit their growth, in order to select their neighbours and interact with them, to increase disease virulence and progression, promote the establishment of biofilms and ultimately play an important role in the balance of the oral ecosystem (Kuramitsu et al., 2007).

It is also likely that the metabolic products, such as hydrogen peroxide, of one organism have negative effects on other organisms within close proximity. This has been shown to be the case for some Streptococcal species comprising dental plaque biofilms (Loesche et al., 1975, Kuramitsu et al., 2007). However, the production of hydrogen peroxide can also be an advantage to some species, allowing them enhanced

resistance to killing by the host innate immune system (Ramsey and Whiteley, 2009).

1.8 Sources of reactive oxygen species, including hydrogen peroxide and peroxide toxicity.

Reactive oxygen species (ROS) are oxygen-derived molecules, including oxygen radicals [superoxide (O_2•−), hydroxyl (•OH), peroxyl (RO_2•), and alkoxyl (RO•)] and certain nonradicals that are either oxidising agents and/or are easily converted into radicals, such as hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$), and hydrogen peroxide (Bedard and Krause, 2007). Their generation is generally a cascade of reactions that forms superoxide, which then dismutates to hydrogen peroxide. This occurs spontaneously at low pH, or can be catalysed by the enzyme superoxide dismutase (Bedard and Krause, 2007). ROS interact with a large number of molecules such as proteins, lipids, carbohydrates and nucleic acids. These interactions may cause ROS to damage the target molecule, this causing damage to the biological system it is interacting with (Bedard and Krause, 2007).

ROS can be generated as a by-product of the respiratory chain, during aerobic respiration of bacteria. In order that enzymes can transfer electrons to oxygen and subsequently form superoxide anions, the enzymes must be capable of carrying out univalent redox reactions (Imlay, 2003). The respiratory chain enzymes meet this requirement and this is further supported by the finding that normal electron transport generates superoxide and hydrogen peroxide in E. coli (Gonzalez-Flecha et al., 1995, Imlay, 1995). It was initially thought that these ROS simply escaped as intermediates from the respiratory chain, but this has proved not to be the case. Instead, it has been shown that *in vitro*, superoxide and hydrogen peroxide are formed by auto-oxidation of respiratory

dehydrogenases (Imlay, 2003). During normal aerobic respiration, these dehydrogenases use reduced flavin cofactors to transfer electrons to either iron-sulfur clusters or bound quinone within the enzyme (Messner and Imlay, 2002). However, if a reduced flavin collides with oxygen before electron transport, it can transfer the electron onto oxygen instead, thus creating superoxide (Messner and Imlay, 2002). This indicates the tendency of flavins to transfer electrons non-specifically onto any electron acceptor which they make contact with (Imlay, 2003).

ROS generation can also occur as a by-product of biological reactions and sources of production include mitochondria, peroxisomes and cytochrome P-450 (Bedard and Krause, 2007). However, it was discovered in the 20th century that human phagocytes generate ROS in respiratory bursts as a primary function of their enzyme systems when they are stimulated as part of an immune response, such as exposure to bacterial lipopolysaccharide (LPS) during infection (Bedard and Krause, 2007). In 1961, it was shown that this respiratory burst resulted in the generation of hydrogen peroxide (Iyer et al., 1961), but it was later shown that the initial product of this burst was in fact superoxide (Babior et al., 1973).

The enzyme responsible for the production of superoxide is NADPH oxidase (NOX) and the catalytic subunit of the phagocyte NADPH oxidase was cloned and named as NOX2 (Royker-Pokora et al., 1986). Alongside these findings, observations were made that suggested these enzyme systems were present in many other cell types and it is now known that such systems exist in virtually all human tissues (Bedard and Krause, 2007). The availability of the human genome sequence enabled research into these systems and subsequently, NOX2 homologues have been identified and are known as NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2 (Bedard and Krause, 2007).

All NOX family members are transmembrane proteins that transport electrons across biological membranes to reduce oxygen to superoxide. In accordance with this preserved function, there are conserved structural

properties of NOX enzymes that are common to all; All NOX family members share six highly conserved transmembrane domains. Transmembrane domains III and V each contain two histidines, spanning two asymmetrical hemes. The cytoplasmic COOH terminus contains conserved flavin adenine dinucleotide (FAD) and NADPH binding domains (Bedard and Krause, 2007).

The activation of NOX occurs through a complex series of protein/protein interactions, resulting in phosphorylation and translocation of the NOX complex to the cell membrane. The final step involves the GTPase Rac, which interacts with NOX via a two-step mechanism (Bedard and Krause, 2007). Once assembled, the complex is active and can begin to generate superoxide.

NOX can be regarded as a transmembrane redox chain that connects the electron donor, NADPH, on the cytosolic side of the membrane with the electron acceptor, oxygen, on the outer side of the membrane (Bedard and Krause, 2007). In the first step, electrons are transferred from NADPH to FAD, follwed by the transfer of a single electron from the reduced flavin FADH2 to the iron center of the inner heme (Bedard and Krause, 2007). Since the iron of the heme can only accept one electron, the inner heme must donate its electron to the outer heme before the second electron can be accepted from the now partially reduced flavin, FADH (Bedard and Krause, 2007). To create an energetically favorable state, oxygen must be bound to the outer heme to accept the electron, forming the superoxide radical (Cross and Segal, 2004).

As discussed previously, superoxide dismutates into hydrogen peroxide, either spontaneously, particularly at low pH, or facilitated by superoxide dismutase (Bedard and Krause, 2007). It is therefore reasonable to predict that killing by hydrogen peroxide derived from superoxide is a function of NOX activity.

Organic peroxides are also produced in human tissues and may have a bactericidal effect. These kinds of peroxides arise *in vivo* as lipid peroxides produced by cyclooxygenase and lipoxygenase activities, via heme iron, to generate reactive and damaging alkoxyl (RO) and peroxyl (RO_2) radicals (Gutteridge and Quinlan, 1993). An earlier study showed that the major cytotoxic radical produced was the alkyl peroxyl radical, ROO – (Akaike et al., 1991). Such radicals have been reported to be involved in haemolysis and cancer promotion (Akaike et al., 1991).

The most important finding from the study by Akaike et al. was the observation that the radicals produced by organic peroxides in humans had a bactericidal effect, particularly on Gram positive bacteria such as *Streptococcus aureus* (Akaike et al., 1991). This indicates that bacteria are exposed to both oxidative stress from hydrogen peroxide derived from superoxide and to organic peroxides and their radicals and these stresses may be encountered during bacterial colonisation of the oral cavity.

The damage caused by superoxide radicals and hydrogen peroxide can target a variety of molecules within the cell. Damage to iron-sulfur clusters within the active site of enzymes can lead to catabolic and biosynthesis defects (Imlay, 2008). Enzymes, such as dehydratases, contain $[4Fe\text{-}4S]^{2+}$ clusters which are oxidised to inactive forms following exposure to superoxide or hydrogen peroxide at the active site (Imlay, 2008). Superoxide anions bind the critical catalytic iron atom within these clusters and univalently oxidise them to the $[4Fe\text{-}4S]^{3+}$ form, which releases the catalytic iron atom and renders the enzyme inactive (Imlay, 2008). The iron released from these reactions has been shown to indirectly damage DNA (Keyer and Imlay, 1996). Hydrogen peroxide oxidises the iron-sulfur cluster divalently, releasing ferric iron and resulting in the $[3Fe\text{-}4S]^{+}$ cluster form (Imlay, 2008).

Both superoxide and hydrogen peroxide are mutagenic (Imlay, 2008) and are therefore capable of damaging aromatic amino acids and subsequently proteins, although the biochemical mechanism for this is not

yet known (Imlay 2003, Imlay, 2008). However, it is hydroxyl radials that can directly damage most biological molecules (Imlay, 2003) and these radicals are formed as an oxidising product of the Fenton reaction, when electron transfer from ferrous iron to hydrogen peroxide takes place, as shown in the equations below (Imlay, 2003, Imlay, 2008);

$$Fe^{2+} + H_2O_2 \rightarrow FeO_2^{+} + H_2O. \qquad 1.$$

$$FeO^{2+} + H^{+} \rightarrow Fe^{3+} + HO^{\bullet}{}_2.$$

The hydroxyl radical acts at almost diffusion-limited rates to oxidise molecules near the site of its formation (Imlay, 2008). The Fenton reaction has been linked to protein carbonylation and membrane peroxidation, but its most significant impact is upon DNA, where small amounts of intracellular hydrogen peroxide can cause lethal DNA damage (Imlay, 2003, Imlay, 2008).

1.9 *Neisseria meningitidis* and its prevalence in the human oral cavity.

The genus *Neisseria* comprises mainly of non-pathogenic species, such as *N. sicca* and *N. flavescens*, which are commonly identified as members of the oral bacterial community, as well as in the upper respiratory tract (Aas et al., 2005). *N. meningitidis* is a pathogenic member of this genus which is also found in the oral cavity and upper respiratory tract in up to one quarter of the population.

Neisseria meningitidis is a Gram-negative coccus which is a pathogenic member of the Neisseriae family and the most common cause of meningitis outbreaks. The natural habitat of *N. meningitidis* is the human nasopharynx, from where the organism may invade the bloodstream, causing bacteraemia leading to meningitis, depending on the virulence of the meningococcal strain (Ala'Aldeen et al., 2000). The highest attack rates of invasive meningococcal disease in the United Kingdom are in the first year of life and then among teenagers, whereas the highest carriage rate is found among teenagers and young adults, which may account for outbreaks seen at Universities (Ala'Aldeen et al., 2000). It was previously thought that the proportion of humans with asymptomatic meningococcal carriage of the nasopharynx was around 10 %, but this has been shown to be underestimated and may be as high as 25 % (Sim et al., 2000).

Meningococcal carriage is usually asymptomatic and it has been shown that colonisation of the nasopharynx by meningococci produces an antibody response from the three major immunoglobulin classes, which may act as an immunising event, occuring a few weeks after initial colonisation (Kremastinou et al., 1999). In order to colonise the nasopharynx, the meningococcus must first adhere to the mucosal surfaces, then has to utilise the available nutrients, as well as evading the host immune response (Yazdankhah and Cauguant, 2004). Therefore, structures and molecules must be produced by the bacteria to enable

these actions to occur. The first of these structures, required to facilitate adhesion to epithelial cell surfaces, are pili. *N. meningitidis* initiates adhesion by attaching to host cells via type IV pili, which are long hair-like structures that extend from the bacterial surface (Merz et al., 2000). A membrane cofactor protein, CD46, which is expressed on all human cells except erythrocytes, has been identified as a receptor for Neisserial type IV pili (Johansson et al., 2003). These pili are involved at multiple stages of colonization, in complex host cell interactions and responses (Yazdankhah and Caugant, 2004).

Once the meningococci have adhered to the membranes of the nasopharynx, they must begin to source nutrients and utilize them in order to survive. *N. meningitidis* expresses receptors which are capable of binding to human transferrin and lactoferrin, which are both thought to be sources of iron and a basic component essential for growth (Larson et al., 2002). The iron acquisition system of the meningococcus includes several proteins; TbpA and TbpB, which are both transferring-binding proteins, LbA and LpB, both lactoferrin-binding proteins, Hbp, a haemoglobin-binding protein and haemoglobin-haptoglobin-binding protein, Hbp-Hpp (Yazdankhah and Caugant, 2004). These proteins bind to human iron-carrying proteins, releasing and internalising iron into the bacterium (Yazdankhah and Caugant, 2004).

The major virulence determinant of *N. meningitidis* is its polysaccharide capsule, which contains both sialic and non-sialic acids (Swartley et al., 1997). This capsule is absent from non-pathogenic strains and is an essential mechanism of survival in the bloodstream, as the capsule has anti-phagocytic properties which enables it to grow in the bloodstream, where it causes septicemia and meningitis (Spinosa et al., 2007). However, the presence of the capsule inhibits the colonisation and invasion of the nasopharyngeal tissues (Spinosa et al., 2007) and it has been previously shown that capsule biosythesis is downregulated during the early stages of infection (Deghmane et al., 2002), which presumably facilitates the adhesion and invasion of host tissues.

In order to evade the host immune response, *N. meningitidis* also expresses an extracellular IgA1 protease coded for the by *iga* gene, which has the capacity to cleave Human IgA1, the dominant immunoglobulin in immune secretions (Lomhalt et al., 1992). IgA1 has an essential role in mucosal tissues, such as in the nasopharynx, by preventing adherence and colonisation of bacteria, so the ability of *N. meningitidis* to cleave this immunoglobulin has importance in adherence also (Yazdankhah and Caugant, 2004). It is interesting to note that the three most common causative agents of bacterial meningitis*, N. meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae*, which all first colonize the nasopharynx (Tunkel & Scheld, 1993), all produce IgA1 protease (Yazdankhah and Caugant, 2004). Furthermore, the ability of *N. meningitidis* to switch its capsule composition provides a further mechanism for evading the host response and presents a difficulty to overcome when designing vaccines against the bacteria (Swartley et al., 1997).

In order to protect Humans from the threat and severity of meningococcal disease, several studies have been carried out to investigate the effect of vaccination on meningococcal carriage, using polysaccharide vaccines, polysaccharide-conjugate vaccines and the serogroup B outer membrane vesicle (Yazdankhah and Caugant, 2004). In general, most meningococcal polysaccharide vaccines are poor immunogens in infants and fail to induce immunological memory in people of any age (Yazdankhah and Caugant, 2004). Furthermore, the effect of the polysaccharide vaccines on colonisation and transmission of the organisms are transient or negligible (Jodar et al., 2002). Local immunity against *N. meningitidis* has been observed in the nasopharynx after vaccination, but levels of antibodies delined rapidly over 6 – 12 months, indicating that the local protection does not last for a long period of time (Yazdankhah and Caugant, 2004), making long term protection difficult to achieve. Development of a vaccine against serogroup B meningococci, such as the *N. meningitidis* MC58 strain used in this study has proved to be difficult because the serogroup B polysaccharide is poorly

immunogenic in humans (Pollard & Frasch, 2001, Yazdankhah and Caugant, 2004).

1.10 *Streptococcus pneumoniae* – Carriage, pathogenicity and hydrogen peroxide production.

Streptococcus pneumoniae are Gram positive, lancet-shaped bacteria that grow in pairs or small chains (AlonsoDeVelasco et al., 1995). They can be found colonising the human respiratory tract with potential to become pathogenic if in high enough numbers. The surface of these bacteria can be distinguished by three major layers – the plasma membrane, the cell wall and the capsule (AlonsoDeVelasco et al., 1995). The cell wall consists of a triple-layered peptidoglycan backbone that anchors the capsular polysaccharide (which is serotype-specific), the cell wall polysaccharide and possibly also proteins (AlonsoDeVelasco et al., 1995). The capsule consists of high-molecular-weight polymers made up of units of repeating oligosaccharides which in themselves can contain between two and eight monosaccharides (AlonsoDeVelasco et al., 1995).

S. pneumoniae is carried in the upper respiratory tract by many healthy individuals. It has been suggested that attachment of the pneumococci to the cells of the nasopharyngeal mucosa is mediated by a disaccharide receptor on fibronectin (AlonsoDeVelasco et al., 1995). It is thought the pathogenicity of *S. pneumoniae* is caused by various structures, situated on its surface, although this and the high morbidity and mortality caused by this microorganism are still poorly understood (AlonsoDeVelasco et al., 1995).

This potential pathogen is able to evade the host immune response due to its capsule providing resistance to phagocytosis (AlonsoDeVelasco et al., 1995). This is an important property required for the pathogenicity of the bacteria, as discussed also in section 1.8 previously. *S. pneumoniae* infection causes significant inflammation in the host and it is thought this inflammation is mediated by virulence factors comprising cell wall components and the intracellular toxin pneumolysin (AlonsoDeVelasco et al., 1995). Since inflammation is thought to induce most of the symptoms

of pneumococcal disease, this group of virulence factors may be more directly responsible for the morbidity and mortality caused by *S. pneumoniae* once it has infected the host.

Another potential contributor to the pathogenicity of some strains of *S. pneumoniae* is their ability to produce hydrogen peroxide (Annear and Dorman, 1952). The enzyme responsible for the production of this toxic compound is pyruvate oxidase, SpxB, which decarboxylates pyruvate to acetyl phosphate plus hydrogen peroxide and carbon dioxide, as a by-product of aerobic metabolism (Pericone et al., 2000, Spellerberg et al., 2003, Regev-Yochay et al., 2007). Although it is known that Streptococcus species are the major hydrogen peroxide producers in the human oral cavity, free hydrogen peroxide levels can not be readily detected in plaque or dental sediments (Ryan and Kleinberg, 1995). This is possibly due to the bias of hydrogen peroxide destruction over production, carried out by bacteria co-colonising the oral cavity (Ryan and Kleinberg, 1995).

Attempts at the beginning of the 20th century to induce immunity in humans against *S. pneumoniae* by vaccination, using whole killed pneumococci were not very successful and the approach was abandoned because of the adverse side effects caused by the large amounts of inocula used (AlonsoDeVelasco et al., 1995).

Investigations in this study were performed with a type strain of *Streptococcus pneumoniae*, as a know hydrogen peroxide producer was required and this species has been used previously in studies involving microbes that inhabit the Human nasopharynx (Pericone et al., 2000, Selva et al., 2009), with the study be Pericone et al. performing experiments under similar conditions and using similar methodologies to those used in this study. The strain chosen, ATCC 6303, has previously been used by Bolm et al. in 2004, where its ability to kill the nematode *Caenorhabditis elegans* was shown to be hydrogen peroxide-mediated, with the strain producing 1.7 mM hydrogen peroxide (Bolm et al., 2004).

1.11 The oxidative stress response of *Neisseria*.

The oxidative stress response system of *Neisseria* has been investigated in both *N. meningitidis* and *N. gonorrheae* (Seib et al., 2007, Ieva et al., 2008). There are several genes involved in this stress response system, with the regulation of each one playing a role in the peroxidases expressed under different levels of oxidative stress.

1.11.1 Catalase.

Catalases, widespread in aerobic bacteria, are heme-cofactored enzymes that convert hydrogen peroxide to oxygen and water (McCord et al., 1971, Sieb et al., 2006). In *Neisseria*, these enzymes are encoded by the *katA* gene, which is regulated by the OxyR protein (Seib et al., 2007, Ieva et al., 2008). It has been shown that levels of catalase are around 100-fold higher in *N. gonorrhoeae* than in *N. meningitidis* and this is thought to be due to the environments colonised by the gonococcus (Archibald and Duong, 1986).

1.11.2 OxyR.

OxyR, widely known as a mediator of hydrogen peroxide-induced gene expression, is a redox-sensitive, tetrameric DNA-binding protein, which shows homology to the LysR regulatory proteins of *E. coli* and NodD of Rhizobium (Christman et al., 1989, Mongkolsuk and Helmann, 2002, Sainsbury et al., 2010). The molecular basis for the redox regulation of OxyR has been investigated in a number of studies, using structural, biochemical, genetic and physiological methods (Mongkolsuk and Helmann, 2002).

Early experiments in *Escherichia coli* indicated that reduced OxyR binds to two adjacent major grooves separated by one helical turn, while in its oxidised form, it binds four adjacent major groove regions and activates transcription by direct contact with RNA polymerase (Mongkolsuk and Helmann, 2002). The initial reaction of OxyR with hydrogen peroxide is thought to occur at the Cys-199 residue, oxidising this and leading to the formation of an unstable Cys–sulphenic acid (Cys–SOH) intermediate (Zheng et al., 2001).

Once oxidised, Cys-199 reacts with the Cys-208 residue to form an intramolecular disulphide bond and it is thought oxidation of these two residues is accompanied by refolding of a central domain of OxyR (Choi et al., 2001).

In *E. coli*, OxyR regulates in excess of ten genes, including genes involved in peroxide metabolism and protection (*katG*, *ahpC*, *ahpF*, *dps*), redox balance (*gor*, *grxA*, *trxC*) and important regulators such as *fur* and the small RNA *oxyS (Zheng et al., 2001)*. The Neisserial OxyR, which shares 37% amino acid sequence identity with the *E. coli* protein, appears to control expression of a smaller regulon comprising the genes encoding catalase (*katA*), glutathione oxidoreductase (*gor*) and peroxiredoxin oxidoreductase (*prx*) (Seib et al., 2007).

In contrast to the findings in *E. coli*, where OxyR is thought to act as an activator of transcription, it has been shown that in both *Neisseria gonorrhoeae* and *Neisseria meningitidis*, *katA* expression is repressed by the regulator OxyR, which is only activated to derepress *katA* expression in the presence of high level peroxides (Seib et al., 2007, Ieva et al., 2008). It has been shown in *N. gonorrhoeae* that an *oxyR* mutant is highly resistant to hydrogen peroxide stress, due to a constitutive de-repression of *katA* caused by the lack of OxyR, leading to continuous high-level catalase production (Tseng et al., 2003).

1.11.3 Peroxiredoxin (Prx).

A further group of peroxidases, capable of breaking down organic peroxides, is Prx. This group of peroxidases lack heme, but catalyse peroxide reduction via cysteine residues (Chae et al., 1994, Rouhier and Jacquot, 2003). Phylogenetic and biochemical analyses indicate that animal, yeast and plant cells contain several Prx (Rouhier and Jacquot, 2003). All Prx contain one conserved cysteine residue which, upon catalysis, is transformed into a sulfenic acid, subsequently regenerated by a thiol reductant, in general thioredoxin (Trx) (Chae et al., 1994). However, this regeneration is more efficiently performed by glutaredoxin (Grx), which is a related catalyst with a modified active site (Rouhier and Jacquot, 2003). Grx is reduced via NADPH, glutathione reductase and reduced glutathione (GR and GSH, respectively) (Rouhier and Jacquot, 2003).

In order to investigate the interaction between Prx and Grx, hybrid enzymes containing both of these domains were studied in both *H. influenzae* and *N. meningitidis* (Vergauwen et al., 2003, Rouhier and Jacquot, 2003). Rouhier and Jacquot showed that fusion sequences from several species shared the same organisation – Prx being present in the N-terminus domain and Grx being present in the C-terminal domain (Rouhier and Jacquot, 2003).

Vergauwen et al. (2003) reasoned that the Prx/Grx fusion represented an important role in peroxide breakdown in *H. influenzae*, as the *prx/grx* gene gene product seemed to be transcribed from a promoter which overlapped with the *oxyR* promoter and hence predicted that the regulation of *prx/grx* expression was linked to OxyR levels present in the cell. This study also showed that these fusions, when expressed in *E. coli*, could complement peroxide negative strains and restore their peroxidase function (Vergauwen et al., 2003).

In *N. meningitidis*, the Prx/Grx fusion protein is able to reduce various hydroperoxides, as well as hydrogen peroxide. These reactions are driven by reduced glutathione which is maintained reduced via NADPH and glutathione reductase (Rouheir and Jacquot, 2003). The Grx domain has been shown to possess a Grx activity and that reduced glutathione is an excellent donor to Prx to catalyse the breakdown of hydrogen peroxide and other organic peroxide radicals (Rouhier and Jacquot, 2003).

In addition to the repression of *katA* expression in wild type *N. gonorrheae*, OxyR may also activate the *prx* gene (Seib et al., 2007), outlining a dual role for OxyR in the meningococcal oxidative stress response. Further to this, it has been reported for *N. gonorrhoeae* that in a *prx* mutant, hydrogen peroxide resistance is elevated due to accumulated hydrogen peroxide allowing de-repression of *katA*, which is presumably mediated by OxyR (Seib et al., 2007). This may outline a role for Prx as as low-level peroxide scavenger in the meningococcus.

1.11.4 Bacterioferritin co-migratory protein (Bcp).

A further peroxidase involved in the oxidative stress response of *Neisseria* is bacterioferritin co-migratory protein (Bcp). Bcp is related to alkylhydroperoxidase (AhpC), which is found in many organisms. AhpC is representative of a very large and ubiquitous family of cysteine-based peroxidases, now designated as peroxiredoxins (Prxs) (Poole, 2005). Both AhpC and Bcp are thiol peroxidases with a conserved cysteine residue at the active site and depend on thioredoxin (Trx) as reductant for their activity (Wang et al., 2006). AhpC was primarily associated with the detoxification of organic peroxides, however, the range of organic peroxides that are good substrates for the enzyme indicates that its active site can accommodate virtually any peroxide, so it is not surprising to learn that this enzyme can also break down hydrogen peroxide (Seaver and

Imlay, 2001). Supporting this are the results from experiments in *Helicobacter pylori*, where AhpC was shown to be able to break down a range of peroxides, including hydrogen peroxide, but its major function is to break down organic peroxides (Wang et al., 2006). It is, however, thought that the role of Bcp in the oxidative stress response of *H. pylori* is minimal, as bcp mutants in this species are only slightly more sensitive to oxidative stress (Comtois et al., 2003). It is thought that the role of Bcp in *H. pylori* may be to break down organic peroxides produced both from lipid metabolism and general oxidative stress (Comtois et al., 2003).

The AhpC system differs slightly in some bacteria, where the reductant for activity is not thioredoxin, but a flavoprotein known as AhpF. This protein is only expressed in bacteria and is a dedicated AhpC reductase in the species which encode it (Poole, 2005). AhpF achieves the direct transfer of electrons from NADH to AhpC via three redox centres, with electron transfer among these three centres involving large domain movements (Poole, 2005). However, given the distinct requirements for the reducing partners of AhpC, the mechanistic and structural properties of the AhpC enzymatic system are extremely similar in most organisms (Poole, 2005).

In *E. coli*, AhpC is a member of the OxyR regulon, as it is in a range of diverse bacteria (Seaver and Imlay, 2001). It has been shown in *E. coli* that AhpC is responsible for the degradation of low-level hydrogen peroxide and is responsible for peroxide scavenging in a catalase-deficient mutant (Seaver and Imlay, 2001). These results indicate that Bcp may have a similar role in *Neisseria* and may play a role in scavenging organic peroxides produced by human tissues.

1.11.5 Glutathione peroxidase (GpxA).

Members of the glutathione peroxidase family are found in many eukaryotes, including humans and other mammals, plants, and parasitic worms, although they are mainly studied in mammals, where they are thought to provide protection from, and to repair damage caused by ROS (Criqui et al., 1009, Brigelius-Flohe et al., 1994). This peroxidase is very limited in the prokaryotes, but it is known to exist in *E. coli*, *N. meningitidis* and *Streptococcus pyogenes* (Arenas et al., 2010). The most extensively studied homolog of this peroxidase BtuE (encoded by the *btuE* gene) from *E. coli*, which was isolated and identified as part of the vitamin B12 transport operon (Moore and Sparling, 1995, Arenas et al., 2010).

Glutathione peroxidase enzymes catalyse the reduction of peroxides, with reduced glutathione acting as the reducing donor (Moore and Sparling, 1995). Two distinct types of these enzymes have been well characterised - a tetramer which reduces both hydrogen peroxide and low-molecular-weight water-soluble hydroperoxides (Brigelius-Flohe et al., 1994) and in contrast, a phospholipid glutathione glutathione peroxidase that is a monomeric enzyme with a similar kinetic mechanism (Moore and Sparling, 1995).

In *Neisseria*, this peroxidase is encoded by the *gpxA* gene, which has been shown to be present in *N. meningitidis* but is distinctly absent from *N. gonorrhoeae* (Moore and Sparling, 1995). It has been shown that inactivation of the *gpxA* gene in *N. meningitidis* results in hypersensitivity to oxidative stress. A *gpxA* insertion mutant has been shown to be considerably more sensitive to peroxide generated by paraquat, but only slightly more sensitive to damage caused by hydrogen peroxide (Moore and Sparling, 1996). This indicates that the GpxA protein may play an important role in the lifestyle and / or pathogenicity of *N. meningitidis* and this study aims to investigate this potential role further.

1.11.6 Superoxide dismutase (SOD).

This class of enzyme catalyses the breakdown of superoxide to hydrogen peroxide and water (Sieb et al., 2006). Three main classes of SOD exist, SodA, SodB and SodC, with SodA and SodB being cytoplasmic and SodC being periplasmic and all are found in *E. coli* (Sieb et al., 2006, Imlay and Imlay, 1996). SodB in *E. coli* has a limited role in protection against oxidative stress, which is restricted to the transition from anaerobic to aerobic conditions (Kargalioglu and Imlay, 1994, Seib et al., 2006). Genes encoding SodA or SodC are not present in *N. gonorrhoeae*, but the gene encoding SodB (the *sodB* gene) is present in the gonococcal genome (Sieb et al., 2006).

In contrast to *N. gonorrhoeae*, the closely related organism *N. meningitidis* contains active SodB and SodC enzymes (Archibald and Duong, 1986, Kroll et al., 1995, Seib et al., 2004). Unlike *N. gonorrhoeae*, SodB of *N. meningitidis* has been demonstrated to play a role in protection against oxidative stress (Seib et al., 2004). The *sodC* gene of *N. meningitidis* is absent in *N. gonorrhoeae* and appears to have been acquired by *N. meningitidis* via horizontal transfer, most likely from commensal *Haemophilus* species that coinhabit the upper respiratory tract (Kroll et al., 1995).

Although all *N. gonorrhoeae* strains contain the *sodB* gene (Tseng et al., 2001), 80%–100% of strains have no Sod activity, and the remainder have very low activity (Archibald and Duong, 1986, Norrod and Morse, 1979). In 2004, Seib et al. showed that *N. meningitidis* had a 3.5-fold higher level of Sod activity than did *N. gonorrhoeae* (Seib et al., 2004). Furthermore, the *N. meningitidis sodB::kan/sodC::tet* mutant strain showed a low residual level of Sod activity, indicating that another mechanism is present that is capable of providing Sod activity in the meningococcus (Seib et al., 2004). It was also shown that the *N. meningitidis sodB::kan* and the *sodC::tet* mutant strains had activity similar to wild type levels, indicating

that both SodB and SodC are highly active and may be up-regulated to compensate for the absence of the other enzyme (Seib et al., 2004). This may also serve as an important role for SODs in response to oxidative stress encountered by the meningococcus during its infective stage and highlights the importance of the oxidative stress response in the lifestyle of the meningococcus.

1.12 Aims of the study.

The first aim of this study wasto identify the bacterial species comprising the oral microflora of one subject, by performing a systematic evaluation of bacterial diversity using both molecular and culture-based methods. RFLP and FAME profiling analyses were carried out simultaneously, with samples originating from whole mouth rinses, consisting of sterile water. Analyses were carried out over a long period of time (approximately 1 year), in order to gain an insight into the stability of the oral microflora and the presence of dominant genera and species. The FAME profiling methodology was also developed to assess whether this technique could be used, as it is for soil populations, to assess microbial diversity using direct FAME detection from a mixed population, thus removing the need for prior culturing and the bias this may create. It was hoped that a large set of data would be generated, defining prevalent genera and providing evidence for a stable microflora over time.

It is reasonable to presume that interactions between members of the oral microflora would occur and thus, following the systematic evaluation of microbial diversity within the oral cavity, experimentsperformed to investigate the potential interactions of the prevalent genera identified, the strength of these interactions and the possible causes / mediators. Initial experiments focused on co-culture of the prevalent genera in both solid and liquid phases, to investigate whether any interactions were apparent. Studies then focused on possible causes and mediators of these interactions, in terms of metabolic by-product detection and gene expression levels during single species and co-culture experiments.

It was hoped that the outcomes of these experiments would not only give an insight into the microbial diversity of the entire oral cavity, but also an insight into the presumably complex interactions occurring between oral microbes in response to co-habiting the same environment.

2. Materials and methods.

2.1 Chemicals.

All chemicals were sourced from Sigma Aldrich, St Louis, Missouri, USA, unless otherwise stated.

2.2 Mouth rinse and salad wash sampling.

Mouth rinses consisted of 10 ml sterile de-ionised H_2O, washed around the mouth in the style of a dental mouthwash for 30 seconds and returned to a sterile tube, with 100 µl being taken for further analysis. There was no deviation from normal oral hygiene and all samples were taken approximately 2 hours after the last time the teeth were cleaned and no food was consumed in this period.

Salad samples, weighing 10 g each, were taken and placed in a solution of 100 ml sterile x1 Phosphate Buffered Saline (PBS) and 2 ml sterile TWEEN 20. Samples were then placed on a Bibby Stuart STR9 gyrorocker and rotated at 70 rpm for 2 hours at room temperature. A sample of 100 µl was then taken for further analysis.

2.3 Restriction fragment length polymorphism analysis (RFLP).

Samples of 100 µl were taken from mouth rinses or salad washes (as described in 2.2) and mixed with 400 µl lysis buffer (50 mM Tris, 1.25 mM EDTA, 1 mM TWEEN 20, pH 7.6), followed by the addition of 5 µl of a 20 mg/ml proteinase K solution and heating at 55 °C for 2 hours, followed by 5 mins at 95 °C. Samples were then centrifuged at 13 000 rpm for 2 minutes at room temperature in a Sigma 113 bench-top microcentrifuge. Supernatants were then used as template DNA in PCR reactions, made up in volumes of 25 µl consisting of 5 µl Go Taq 5x flexi buffer (Promega), 1.5 µl $MgCl_2$ (25mM), 2.0 µl dNTPs (2.5 mM each), 0.375 µl each primer (20 pmol/µl), 0.2 µl Go Taq DNA polymerase (Promega), 14.55 µl molecular biology grade H_2O (Sigma) and 1 µl template DNA. Primers used were the universal bacterial primers 27f (5' GAG TTT GAT YMT GGC TCA G 3') and 1492r (5' TAC GGY TAC CTT GTT ACG ACT T 3') (Lane, 1991). The PCR programme used was 94 °C 2 minutes, followed by 30 cycles of 94 °C 15 sec, 52 °C 30 sec, 72 °C 2 min and a final elongation step of 72 °C for 5 mins. Products were viewed on an 0.8 % agarose gel containing 0.21 ng/ml ethidium bromide, using a Syngene Gene Genius UV transilluminator gel documentation system. Bands were excised using a sterile scalpel and stored in pre-weighed Eppendorf tubes. DNA was then extracted using a QIAGEN QIAquick gel extraction kit (QIAGEN), according to the manufacturer's instructions.

Purified PCR products were subsequently cloned into a pGemT-easy vector (Promega), in a reaction volume of 10 µl, consisting of 5 µl 2x ligation buffer (Promega), 3 µl pure DNA, 1 µl T4 DNA ligase (Promega) and 1 µl pGemT-easy vector (Promega). All reactions were incubated overnight at 4°C before being transformed into chemically competent *E. coli* DH5α cells. The cloning reaction was gently mixed with 200 µl *E. coli* DH5α cells and incubated on ice for 20 minutes, before being heat shocked at 42 °C for 50 seconds, then cooled on ice for 2 minutes. Following this, 500 µl sterile lysogeny broth (LB) was added and cells were

cultured for 2 hours at 37 °C, with shaking at 200 rpm on an Innova 2000 platform shaker (New Brunswick Scientific). Samples were then centrifuged for 5 minutes at 6000 rpm in a Sigma 113 bench top microcentrifuge and pellets resuspended in 50 µl sterile LB broth. The resulting cell suspensions were plated out for blue/white selection on LB agar plates containing 100 µg/ml ampicillin, 100 µM IPTG and 100 µM X-Gal. Plates were incubated at 37 °C overnight and positive transformants were identified and transferred to fresh selection plates for further growth and screening.

Template DNA was prepared from positive transformants by suspending a colony in 50 µl of molecular biology grade water (Sigma) and heating at 95 ºC for 10 minutes. Samples were then centrifuged at 13 000 rpm for 2 minutes in a Sigma 113 bench top microcentrifuge. The resulting supernatant was used as template DNA in PCR reactions (as described above). Products were purified using the QIAGEN QIAquick PCR purification kit (QIAGEN), according to the manufacturer's instructions. Aliquots, 2 µl each, of the purifications were viewed using gel electrophoresis as described above.

Restriction digestion reactions were carried out on positive transformants in volumes of 10 µl, consisting of 6 µl molecular biology grade H2O (Sigma), 1 µl buffer C (Promega), 0.5 µl Hha1 restriction enzyme (Promega) and 2.5 µl purified PCR product. Reactions were incubated overnight at 37 °C, before being separated on a 1.5 % agarose gel containing 0.21 ng/ml ethidium bromide and visualised as described above. Distinct phylotypes were identified and cross-referenced to the colony they originated from. Colonies were cultured overnight in 5 ml LB broth containing 100 µg/ml ampicillin in Universal tubes at 37 °C, with shaking at 200 rpm on an Innova 2000 platform shaker (New Brunswick Scientific). For plasmid preparation, 3 ml of the culture was used,using the QIAGEN QIAprep spin miniprep kit (QIAGEN), according to the manufacturer's instructions. Plasmid preparations were eluted in 30 µl of QIAGEN elution buffer and their concentrations determined using a

Nanodrop spectrophotometer. Samples containing more than 100 ng/µl DNA were prepared for sequencing in premixed reactions containing DNA (final concentration 20-40 ng), 1 µl 27f primer (3.2 µM) and molecular grade H_2O, in a final reaction volume of 7.75 µl. Sequencing reactions were carried out on an ABI 3130 DNA sequencer (Applied Biosystems) and viewed using the ABI Sequence Scanner 1.0 software (Applied Biosystems). Poor quality data were trimmed from the ends of sequences to ensure accurate BLAST identifications. Sequences were then compared to those in the BLAST database, using the BlastN nucleotide BLAST search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

A sequence identity match of minimum 97 % was used to determine species level identity (Stackenbrant and Goebel, 1994).

2.4 Fatty acid methyl ester profiling (FAME).

Samples, 100 µl each, were taken from mouth rinses or salad washes (as described in 2.2). Salad washes were plated on nutrient agar plates (NA) and mouth rinses were plated on HBA plates (prepared as in 2.9). Anaerobic cultures were prepared by plating samples on brain-heart infusion agar plates supplemented with 5 % horse blood (BHIBLA) in an anaerobic glove box and sealed in an anaerobic gas jar (Oxoid) before incubation. NA plates were incubated overnight at 28 °C and HBA/BHIBLA plates incubated at 37 °C, according to the MIDI protocol for initial colony isolation (MIDI Inc.). Each distinct colony type was then transferred to a fresh plate and re-streaked to purity, before being transferred to the specific media required for FAME profiling, defined by the MIDI protocol (MIDI Inc.). For samples grown on NA, the media was trypticase soy broth agar (TSBA, Beckton, Dickinson & Co.) and for samples grown on HBA, the media was trypticase soy agar supplemented with 5 % sheep blood (TSA-SB, Becton, Dickinson & Co.). Anaerobic cultures were re-streaked onto fresh BHIBLA plates under anaerobic conditions. Cultures were grown in temperature controlled incubators according to the MIDI protocol (TSBA; 28 °C for 48 hours, TSA-SB/BHIBLA; 35 °C for 24 hours), before being harvested.

The following day, approximately 40 mg of bacterial cells were harvested using a sterile disposable plastic 10 µl inoculation loop, placed in a glass test tube and sealed with a Teflon cap. Samples were saponified by adding 1 ml of 'reagent 1' (FPLC grade NaOH + methanol) and heated at 100 °C for 30 minutes, vortexing after 5 minutes. Bacterial fatty acids were then methylated to their fatty acid methyl esters (FAMEs) by addition of 2 ml 'reagent 2' (6M HCl + methanol) and heating at 80 °C for 10 minutes. FAMEs were then extracted by adding 1.25 ml of 'reagent 3' (methyl tert-butyl ether + hexane), followed by 10 minutes of end over end rotation, separating the samples into 2 phases. The aqueous phase was removed and discarded and the remaining sample was washed with 3 ml NaOH and rotated end over end for 5 minutes. Again the samples

separated into 2 phases and saturated NaCl solution was added until the solvent phase cleared. This was carefully removed using a glass Pasteur pipette, transferred to a GC vial and clamped tightly. Samples were analysed on a Hewlett Packard HP6890 series gas chromatograph, through a 25 metre x 0.2 mm coiled capillary column at a rate of 50 ml/minute. The carrier gas used was helium. The temperature was ramped from 170 °C at rest, to 290 °C at the end of the analysis, with the whole analysis controlled by the HP Chemstation software. Interfacing with Midi Sherlock software, FAMEs were compared to those stored in the libraries TSBA6, Clin6 and the anaerobic library (MIDI Inc.) alongside the FERA (Food and Environment Research Agency) library NCPPB3 (FERA, Sand Hutton, York). A commercially available MIDI calibration standard (Agilent Technologies) was also analysed several times during sample analysis to ensure accurate results were obtained.

2.5 FAME profiling from a mixed sample.

Whole mouth rinse samples were analysed without prior culturing, to assess whether diversity of bacterial FAMEs could be observed. Mouth rinse samples, 3.3 ml, were prepared in triplicate, alongside 3.0 g soil samples as controls. FAME preparation took place as described in 2.5, with the following adjustments; double volumes of reagents 1 & 2 were used (2.0 ml and 4.0 ml, respectively), samples were centrifuged at 2500 rpm for 10 minutes after the 80 °C incubation and the end over end rotation period was increased from 10 to 20 minutes, to ensure a more complete extraction and enhanced extraction of low level FAMEs. FAME analysis by gas chromatography took place as described in Section 2.5, comparing samples to the TSBA6 library (MIDI Inc.).

2.6 Bacterial strains and growth conditions.

Initial experiments were performed using isolates obtained during culturing of mouth rinse samples for FAME profiling. The three dominant genera used were *Neisseria sp.*, *Streptococcus sp.* and *Veillonella sp*. (as identified from BLAST searches, as in 2.3). All strains were cultured at 37 °C overnight on Horse Blood Agar (HBA) plates, made by adding 5 % defibrinated horse blood (TCS Biosciences) to cooled molten Columbia Agar Base (Oxoid). Liquid cultures were in Mueller Hinton Broth (MHB) (Oxoid). *Neisseria* and *Streptococcus* strains were cultured under aerobic conditions in 5 ml medium in 25 ml Universal tubes, at 37 °C with shaking at 200 rpm. *Veillonella* plate cultures were prepared under anaerobic conditions in an anaerobic glove box and maintained in an anaerobic gas jar (Oxoid), with the addition of an AnaeroGen sachet (Oxoid) and an anaerobic indicator strip (Oxoid). Liquid cultures of *Veillonella* were maintained in 23 ml MHB in 25 ml Universal tubes, at 37 °C with shaking at 90 rpm.

Overlay assays were performed by culturing 10 µl samples of each isolate in the centre of individual HBA plates at 37 °C in 5 % CO_2 overnight. Liquid cultures for overlaying the HBA plates were cultured as described above.

Type strains from the genera *Neisseria* and *Streptococcus* were obtained. The MC58 strain of *Neisseria meningitidis* serogroup B was used, as the genome has been fully sequenced and annotated (Tettelin et al., 2000). *Streptococcus pneumoniae* ATCC 6303 was purchased from the American Type Culture Collection (Manassas, USA). Both *Neisseria meningitidis* and *Streptococcus pneumoniae* were cultured at 37 °C in 5 % CO_2 overnight on HBA plates. Liquid cultures were as described above with *Neisseria meningitidis* supplemented with 10 mM $NaHCO_3$ and *Streptococcus pneumoniae* supplemented with 210 U/ml catalase. Long

term storage of stock cultures were in 1 ml aliquots of 50 % glycerol in MHB at -80 °C.

2.7 Recombinant *N. meningitidis* strains.

Throughout this study, many previously constructed forms of mutant *N. meningitidis* MC58 were used. The genes of interest involved in the meningococcal response to hydrogen peroxide were inactivated by insertional deletion techniques, by previous members of the Moir laboratory. The *katA*::Kan mutant contains a Kanamycin resistance (Km^R) cassette and was constructed by Dr Karin Heurlier. The *oxyR*::Tet mutant contains a tetracycline resistance (Tet^R) cassette and was also constructed by Dr Karin Heurlier. The *bcp*::Erm strain contains an erythromycin resistance (Erm^R) cassette and was constructed by Dr Kristen Dyet. The *gpxA*::Spc and *prx*::Spc mutants both contain the omega (Ω) cassette encoding spectinomycin resistance (Spc^R) and were constructed by Dr Kristen Dyet and Dr Karin Heurlier. All mutant strains were routinely cultured in the presence of antibiotics in both solid and liquid media, in order to maintain the antibiotic resistance cassettes. All mutants used in this study, along with the amounts of each antibiotic used are shown in Table 2.7.1. The genotype notation used in this table is shortened throughout the main text of this thesis to omit the antibiotic resistance cassette information, in order to simplify the text for the reader.

Name and gene Nomenclatured	Description	Antibiotic Resistance (concentration)	Reference
katA::Kan NMB0216	MC58 with insertion of a Kanamycin resistance cassette to disrupt the katA gene	Kan^R (50 µg/ml)	Not published
oxyR::Tet NMB0173	MC58 with insertion of a Tetracycline resistance cassette to disrupt the oxyR gene.	Tet^R (2.5 µg/ml)	Not published
prx::Spc NMB0946	MC58 with insertion of a Spectinomycin resistance cassette to disrupt the prx gene.	Spc^R (50 µg/ml)	Not published
bcp::Erm NMB0750	MC58 with insertion of an Erythromycin cassette to disrupt the bcp gene.	Erm^R (5 µg/ml)	Not published
gpxA::Spc NMB1621	MC58 with insertion of a Spectinomycin resistance cassette to disrupt the gpxA gene.	Spc^R (50 µg/ml)	Not published
bcp::Erm.oxyR::Tet NMB0750 NMB0173	bcp::Erm mutant (as above) transformed with MC58 oxyR (as above) chromosomal DNA to create a double bcp.oxyR mutant.	Erm^R/Tet^R (5 / 2.5 µg/ml)	This work
bcp::Erm.prx::Spc NMB0750 NMB0946	bcp::Erm mutant (as above) transformed with MC58 grxprx (as above) chromosomal DNA to create a double bcp.prx mutant.	Erm^R/Spc^R (5 / 50 µg/ml)	This work

Table 2.7.1. Mutant *N. meningitidis* strains used in this study.

2.8 Transformation of *N. meningitidis* strains by the TSB method.

Double mutant *N. meningitidis* strains were subsequently constructed by the transformation of existing single mutant strains with chromosomal DNA from other mutant strains via homologous recombination of the *N. meningitidis* chromosome. Transformations were carried out according to a method used by Dr Melanie Thomson (personal communication, 2009). Single mutant strains were cultured from frozen stocks overnight on HBA plates containing the antibiotic required. Aerobic cultures were inoculated as described in Section 2.7 and grown to mid-log phase (OD600 nm = 0.5 – 1) when 2 - 3 ml of cells were harvested by centrifugation at 8000 rpm in a Sigma 113 bench top microcentrifuge. Cell pellets were resuspended in 100 µl of cold transforming storage buffer (TSB) which comprises MHB pH 6.5 with 10 % PEG 4000, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 5 % w/v DMSO, sterilised and stored at -80 °C. Chromosomal DNA (extracted as in 2.10) containing the mutation of interest was sheared by repeat freeze-thaw cycles, before 10 µl was added to the cells in TSB. Samples were incubated on ice for 50 mins before the addition of 1 ml pre-warmed MHB. Cells were then cultured for 1.5 hours at 37 °C, 200 rpm. The cells were pelleted as before and resuspended in 50 µl MHB before spreading onto HBA plates containing the antibiotic to select for the newly introduced resistance cassette. Plates were incubated overnight at 37 °C in 5 % CO_2 and resultant colonies were re-plated onto HBA containing antibiotics for selection of both the primary and secondary resistance cassettes. Successful clones were verified by PCR. All primers used for verifying both single and double recombinant *N. meningitidis* strains are shown in Table 2.8.1.

Name	Sequence (5' – 3')
Nm katA_F	CTGACCCAGTGCGGGAT
Nm katA_R	CGACAATCAAAACAGCCTGA
Nm oxyR_F	TTAACCGAATTGCGGTACATC
Nm oxyR_R	CGCAGATAAAACTTACCCCG
Nm prx_F	GCGTTTGCAAGATCGTACCGGTCAAA
Nm prx_R	CCGGTAATGGCGCGAACGGA
Nm bcp_F	TGAAATATGAATTTACCCTGCC
Nm bcp_R	CGCTTTCCAATACTTCCTGC
Nm gpxA_F	TCTTCGGGCGTAACACTAGG
Nm gpxA_R	AATGAAAGATGCAGAAGGCAA

Table 2.8.1. PCR primers used to verify recombinant *N. meningitidis* strains in this study.

2.9 Extraction of chromosomal DNA.

This method is based on one used previously (Thomson, 2008) and uses phenol:chloroform:isoamylalcohol to extract genomic material and cetyltrimethylammonium bromide (CTAB) to remove contaminants. All solutions were prepared in DNase/RNase free water. *Neisseria* strains were cultured from frozen stocks overnight on HBA plates (as described in 2.9) before being resuspended in 500 µl Tris-EDTA (TE) buffer (10mM Tris, 1 mM EDTA), pH 8.0 in a sterile 2 ml centrifuge tube (Eppendorf, Germany). The cells were then heat-killed at 65 °C for 45 minutes before the addition of 1 % proteinase K, 1.25 U RNase (New England Biolabs, USA) and 100 µg/ml hen egg lysosyme (final concentrations). Samples were mixed gently before incubation overnight at 37 °C in 5 % CO_2.

The following day, 30 µl of 10 % sodium dodecyl sulfate (SDS) solution was added and samples incubated for 1 hour at 37 °C to complete cell lysis. A 10 % CTAB solution was preheated at 65°C and 80 µl was added to samples, along with 100 µl 5 M NaCl, before incubation at 65 °C for 10 mins. Phenol:chloroform:isoamylalcohol (24:25:1) mix (Fluka), 800 µl, was then added and samples were placed on a rotating inverting shaker for 30

mins at room temperature. Samples were then separated by centrifugation at maximum speed in a Sigma 113 bench top microcentrifuge for 15 mins. The clear, upper supernatant was removed and placed in a clean 2 ml tube. The addition of phenol:chloroform:isoamylalcohol, mixing for 30 mins and centrifuging for 15 mins was repeated a further two times. After the third extraction, the clear supernatant was removed into a clean 1.5 ml tube and an equal volume of 100 % isopropanol was added to precipitate the DNA. Samples were centrifuged at maximum speed in a Sigma 113 bench top microcentrifuge for 20 mins and the supernatant was carefully removed and discarded. Pellets were resuspended in 300 µl 70 % ethanol and centrifuged as above for 10 mins. The ethanol was removed and pellets air-dried overnight at 37 °C. Pellets were resuspended in 20 µl TE buffer pH 8.0 and the concentration determined spectrophotometrically by the absorbance at 260 nm (A_{260}). Genomic DNA stocks were then stored at -20 °C and thawed twice before use to shear the DNA.

2.10 RNA extraction and cDNA synthesis.

Total RNA was extracted for real-time PCR (RT-PCR) determination. Cultures were grown as described in section 2.7, until mid-log phase (OD_{600} = 0.5 - 1). RNA was prepared from 3 ml culture samples, which were harvested by centrifugation (as described previously) and pellets resuspended in 1 ml RNA Protect bacterial agent (QIAGEN) before being incubated at room temperature, with vortexing for 10 seconds every 2 minutes. Samples were then centrifuged and the supernatant removed. Pellets were resuspended in 200 µl Tris-EDTA buffer before the addition of 1 mg/ml lysosyme. Samples were incubated at room temperature for 10 minutes, with vortexing for 10 seconds every 2 minutes. RNA extraction was then carried out using an RNeasy mini-kit (QIAGEN), according to the manufacturer's instructions. Samples were quantified using a NanoDrop spectrophotometer. A cDNA copy of the RNA was synthesised by using 1

µg RNA as a template. All reactions were set up in RNAse free eppendorf tubes, in a total reaction volume of 20 µl, consisting of 10 µl RNA extract, 1 µl Random Oligonucleotides (10 mM) and 1 µl dNTP mix (10 mM) and incubated at 65 °C for 5 mins, then on ice for 1 min, before the addition of 4 µl 5x Reverse Transcription buffer, 2 µl DTT (0.1 M) and 1 µl RNaseOut® (40 U/ml) and incubated at 42 °C for 2 mins. Superscript II Reverse Transcriptase (1 µl of concentration 200 U/ml) was then added and the mixture incubated at 42 °C for 2 h. The enzyme was then inactivated at 70 °C for 15 mins, before the cDNA was stored at -20 °C until use in RT-PCR experiments. All reagents used in this method were supplied by Invitrogen.

2.11 Real-time PCR (RT-PCR) analysis of gene expression.

Primers for the desired target genes were designed using the PrimerExpress software (Applied Biosystems, CA, USA) and are listed in Table 2.11.1. The target genes were those involved in the hydrogen peroxide response of *Neisseria* (*katA, oxyR, prx, bcp, gpxA*). An endogenous housekeeping gene, *metK* (S-adenosylmethionine synthetase, NMB1799), was also measured as a negative control. Transcript levels were measured by RT-PCR using Power SYBR Green PCR Master Mix and an ABI 7000 sequence analyser, according to the manufacturer's instructions. Transcript levels were quantified using the Threshold Cycle (C_T) method, relative to the expression of the endogenous negative control gene, *metK*, using the ABI 7000 System Sequence Detection Software version 1.2.3.

Name	Oligonucleotide sequence (5’ – 3’)
Nm oxyR_RT_F	GGGCATTCATTTGAGGAACTG
Nm oxyR_RT_R	TCCGTCAGCAGCAAAACCT
Nm katA_RT_F	TTTGTTGATGTCGTGCGTTAC
Nm katA_RT_R	CCGTATGCACGCCAAAGG
Nm bcp_RT_F	GCGGTGCGGATTTTCATT
Nm bcp_RT_R	CCAAGCCTTCCGTCGTACAG
Nm gpxA_RT_F	CGCGGTTGACGAGGAATTT
Nm gpxA_RT_R	TGCCAGCTTTGGGAGAGAAG
Nm prx_RT_F	CCCTGCCCGGTGCATT
Nm prx_RT_R	GCCGAACAATTCGTTGTAACG
metkRTF	GCGCAAGACCCAAAAGCA
metkRTR	GCCTGCCAATACGCACAA

Table 2.11.1. **Real Time PCR primers used in this work**

2.12 Real-time PCR (RT-PCR) analysis of hydrogen peroxide-stressed *Neisseria*.

The following *N. meningitidis* strains were chosen to investigate changes in gene expression following exposure to hydrogen peroxide stress; Wild-type MC58, *bcp*::Erm, *bcp*::Erm.*oxyR*::Tet and *bcp*::Erm.*prx*::Spc. Bacteria were cultured to mid-log phase (as described in Section 2.7) before being stressed by the addition of 50 µM hydrogen peroxide for 5 mins. Culture samples, 3 ml, were then harvested by centrifugation (8000 rpm, 10 mins) and total RNA prepared from the pellets (as described in Section 2.11). cDNA was then synthesised from the RNA samples (as described in Section 2.11), followed by RT-PCR analysis (as described in 2.12) using the primers listed in Table 2.12.1.

2.13 Bacterial overlay experiments.

Bacterial overlay experiments were carried out to assess interactions between the dominant genera identified (*Streptococcus*, *Neisseria* and *Veillonella*), using the isolates obtained from mouth rinse cultures. The method was developed with all experiments carried out in triplicate. Spots, 10 µl of each isolate, were placed in the centre of HBA plates, with additional streak plates prepared for culture inoculation. Liquid cultures were inoculated to an initial optical density (OD_{600nm}) of 0.05, measured using an Eppendorf Biophotometer spectrophotometer (Eppendorf, Germany). Culture samples, 1 ml each, were measured by OD_{600nm} every hour until mid-log phase, when 200 µl of culture was added to 10 ml of molten 0.3 % Mueller-Hinton agar (MHA), mixed briefly and poured gently over the bacterial spots. Chemical control experiments were also carried out, replacing the bacterial spots with circles of Whatman filter paper soaked with 10 µl hydrogen peroxide (Sigma), at concentrations of 100 mM and 200 mM. All plates were incubated overnight at 37°C in 5 % CO_2 and observed for areas of clearing / increased bacterial growth the following morning.

2.14 Liquid co-culture experiments.

To assess the effect of *S. pneumoniae* on various *N. meningitidis* strains, liquid co-culture experiments were set up as follows; pre-cultures of *S. pneumoniae* and *Neisseria* were cultured (as described in Sections 2.7 and 2.8) until the start of log phase growth. Co-cultures were prepared in 5 ml MHB supplemented with 10 mM $NaHCO_3$ +/- 210 U/ml catalase and cultured at 37 °C with shaking at 200 rpm. Co-culture inocula were standardised by bacterial OD_{600nm}, with 20 µl of each bacterial pre-culture being inoculated. Each hour, 100 µl samples were removed from the co-culture and serially diluted in MHB to final dilutions of 10^{-4} and 10^{-5}. 100 µl samples of each dilution were plated in triplicate on HBA (prepared as in 2.7) and incubated overnight at 37 °C in 5 % CO_2. Species were distinguished by colony morphology the following day, with each type of colony counted and cfu/ml calculations determining bacterial survival.

2.15 Hydrogen peroxide killing assays.

To investigate the sensitivity of *Neisseria* strains to hydrogen peroxide, killing assays were performed on the following strains; MC58, *katA*::Kan, *oxyR*::Tet, *bcp*::Erm, *prx*::Spc, *gpxA*::Spc, *bcp*::Erm.*oxyR*::Tet and *bcp*::Erm.*prx*::Spc. Cultures were prepared (as described in Section 2.7 and 2.8) and the OD_{600nm} measured every hour until mid-log phase was reached. Hydrogen peroxide, 1mM, was then added to the cultures and 100 µl samples taken every 15 mins for 1 hour. Samples were serially diluted in MHB to a final dilution of 10^{-4}, before being plated in triplicate on HBA plates (with relevant antibiotics where necessary, see 2.8). All plates were incubated at 37 °C overnight, in 5 % CO_2. Resulting colonies were counted the following morning and colony forming units/ml (cfu/ml) calculations were performed to assess bacterial survival.

To further investigate the protection of MC58 by increased *katA* expression, MC58 and *katA*::Kan strains were cultured (as described in Sections 2.7 and 2.8) to mid-log phase, where they were pre-treated with 20 µM or 50 µM hydrogen peroxide for 1 hour, before being exposed to 1 mM hydrogen peroxide for a further hour. Samples were taken as described above throughout the 2 hour exposure period.

2.16 Assaying hydrogen peroxide production from *Streptococcus pneumoniae*.

In order to measure the concentration of hydrogen peroxide produced by *S. pneumoniae*, a standard curve was prepared from known concentrations of hydrogen peroxide, diluted in MHB and assayed using a Hydrogen Peroxide Assay Kit (National Diagnostics), according to the manufacturer's instructions. *S. pneumoniae* cultures were then prepared (as described in 2.7) and the OD_{600nm} measured each hour over an 8 hour growth period. Alongside these readings, the concentration of hydrogen peroxide present in the culture was also measured by harvesting 0.5 ml culture samples by centrifugation (8000 rpm, 10 mins) and supernatants assayed as above. Co-cultures of *S. pneumoniae* and various *N. meningitidis* strains were also assayed as described above.

3. Microbial diversity in the human mouth.

3.1 Identification of bacteria isolated from the mouth.

The first aim of the project was to gain an overview of the bacterial species living in the human mouth. Previous studies have identified bacteria from various sites within the mouth, such as plaque scrapings and subgingival crevices (Sakamoto et al., 2005, Paster et al., 2006) whereas this study analysed whole mouth rinses, sampling multiple sites at once. This systematic evaluation of microbial diversity within the mouth is important in establishing the prevalent species to use in further studies, investigating the interactions between them.

3.2 Bacterial identification using restriction fragment length polymorphism (RFLP) analysis.

RFLP analysis is a method in which different length DNA fragments are analysed following restriction digestion. The difference in length between fragments occurs due to the differences in DNA sequences between organisms - restriction enzymes will only cut DNA at their particular recognition site and the differences in the sequence of DNA between recognition sites results in differing lengths of DNA fragments for each individual DNA sample after restriction digestion. The fragments are separated by gel electrophoresis, resulting in a number of specific bands being present for each DNA sample. The pattern of these bands is known as a phylotype and distinct phylotypes can be used to further analyse the DNA sequence in order to identify the organism from which it originated.

In this study, RFLP analysis was initially used to identify bacterial species from whole mouth rinses, in order to evaluate the diversity of the oral

microbial flora. As the 16S rRNA genes are known markers of relatedness between bacterial species (von Witzengerodeet al., 1997), these genes were chosen to be used for the analysis.

To perform this analysis, DNA was extracted from bacteria contained in whole mouth rinses consisting of 10 ml dH_2O, rinsed around the mouth in the style of a dental mouthwash. A sample of the mouth rinse was subjected to cell lysis in a lysis buffer containing Tris, EDTA, TWEEN 20 and proteinase K (see Materials and Methods section 2.2), following which, cell debris was pelleted by centrifugation and the supernatant containing bacterial DNA used as template in proceeding PCR amplifications, where the 16S ribosomal RNA (16S rRNA) genes were amplified using universal bacterial primers (de Lillo et al., 2006). Following amplification, the 16S rRNA gene fragments were isolated and purified, before being cloned into a vector and subjected to restriction digestion with *Hha1* to produce RFLP phylotypes. Each distinct phylotype was sequenced and the results compared to those in the BLAST database, using a BlastN nucleotide search to identify the species from which the 16S rRNA gene was amplified.

Figure 3.1 overleaf shows typical results from RFLP analyses performed on mouth rinses in this study. The DNA fragments have been separated by gel electrophoresis and are present as phylotypes. Each distinct phylotype observed can then be investigated further, to ascertain the bacterial species from which it originated, thus giving an insight into the diversity of microbes present within each mouth rinse analysed.

Figure 3.1.

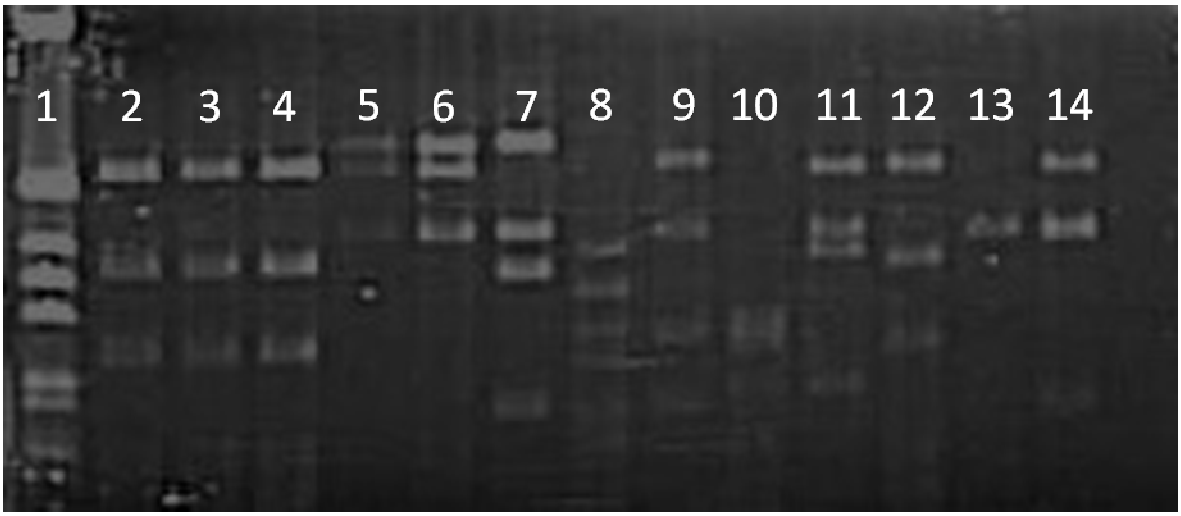


Figure 3.1. A typical RFLP analysis performed on whole mouth rinse samples during this study. 16S rDNA fragments have been digested with the restriction enzyme Hha1, producing phylotypes. Lane 1 contains 1.5 kb DNA ladder (Invitrogen) and lanes 2 – 14 contain individually cloned 16S rDNA fragments digested with HhaI, with nine distinct phylotypes being observed in lanes 2, 5, 7, 8, 9, 10, 11, 12 and 13.

The results in Table 3.1 overleaf were obtained after RFLP analysis, as described above, of a whole mouth rinse. In this analysis, the phylotypes were dominated by the genera *Neisseria* and *Streptococcus*, with over half of the total phylotypes observed belonging to these genera. There were 41 phylotypes that could be assigned to bacterial genera, with 28 of these being distinct.

All genera identified are known to colonise the Human mouth, with *Prevotella*, *Fusobacterium* and *Porphyromonas* all being involved in dental plaque biofilm formation and members of these genera having the ability to be pathogenic (Sakamoto et al., 2005, Kilian et al., 2006).

Table 3.1.

Genus	n	No. distinct phylotypes	Genus description
Neisseria	12	3	Proteobacteria, aerobic diplococci, generally non-pathogenic commensal microbes.
Veillonella	2	2	Firmicutes, anaerobic, cocci, colonise the mouth and respiratory tract.
Prevotella	5	5	Bacteroidetes, anaerobic rods, non-motile, singular cells, colonise the mouth and can be responsible for periodontitis, have natural antibiotic resistance genes.
Streptococcus	13	9	Firmicutes, facultative anaerobic cocci, colonise the mouth and respiratory tract, can cause infections.
Eubacterium	2	2	Firmicutes, anaerobic rod, colonise cavities of humans and animals.
Fusobacterium	2	2	Eubacteria, anaerobic rod, pathogenic, adheres with gram +v and gram –ve microbes in dental plaque biofilms.
Porphyromonas	1	1	Bacteroidetes, anaerobic rods, oral pathogen, hemin used as an iron source for growth.
Gemella	3	3	Firmicutes, anaerobic cocci, closely related to *Streptococcus.*
Actinomyces	1	1	Actinobacteria, anaerobic/facultatively anaerobic, opportunistic pathogens of oral cavity.

Table 3.1. The table shows the genera identified from an RFLP analysis of a whole mouth rinse. Nine genera were identified, with n being the abundance of each genus (i.e. the number of times that phylotypes from the genus were observed).

The results in Table 3.2 overleaf show genus level identification of bacterial diversity in a second, separate whole mouth rinse. The mouth rinse analysed here is completely independent to that which the results in table 1 were obtained from, yet all but one of the genera have been identified previously. In this analysis, 16 identifiable phylotypes were observed and only one of the genera identified had more than one distinct phylotype associated with it. This was *Streptococcus*, which had three distinct phylotypes corresponding to species within the genus. Again, *Streptococcus* and *Neisseria* were dominant genera, with 6 and 4 clone representatives, respectively. This means that out of the 16 clones

analysed, 10 of them were identified to be either *Neisseria* or *Streptococcus*. There was a further distinct phylotype identified, with 16S rDNA sequence matching at a family level only to the Lachnospiraceae.

Table 3. 2.

Genus	n	No. distinct phylotypes	Genus description
Prevotella	1	1	Bacteroidetes, anaerobic rods, non-motile, singular cells, colonise the mouth and can be responsible for periodontitis, have natural antibiotic resistance genes.
Streptococcus	6	3	Firmicutes, facultatively anaerobic, non-motile cocci, colonise the mouth and respiratory tract, can cause infections (tonsilitis).
Veillonella	1	1	Firmicutes, anaerobic, cocci, colonise the mouth and respiratory tract.
Neisseria	4	1	Proteobacteria, aerobic diplococci, generally non-pathogenic commensal microbes.
Corynebacterium	1	1	Actinobacteria, rods, facultatively anaerobic, non-motile, part of skin flora but *C. matruchotti* is an oral pathogen.
Fusobacterium	1	1	Eubacteria, anaerobic rod, non-spore forming, pathogenic, adheres with gram +v end gram –ve microbes in dental plaque biofilms.
Lachnospiraceae	2	1	Sequence identified at family level only.

Table 3.2. This table shows results from an RFLP analysis of a second whole mouth rinse, carried out independently of the rinse giving rise to the results in Table 1. Again n corresponds to the abundance of the genera. Six different genera were identified, with only one of these (*Streptococcus*) having more than one phylotype associated. It can also be seen that with the exception of *Corynebacterium*, all genera have been previously identified by RFLP (see Table 3.1). A further phylotype was identified as originating from the Lachnospiraceae, but the 16D rDNA sequence match was at a family level only.

3.3 Bacterial identification using fatty acid methyl ester (FAME) profiling.

In order to further examine the microbial diversity of the mouth and to utilise and develop an additional technique, Fatty Acid Methyl Ester (FAME) profiling was used to identify bacteria isolated from cultures originating from whole mouth rinses.

As each species of bacteria has a different composition of fatty acids, FAME profiling can be used as a method of bacterial identification. Bacteria are cultured under strict conditions before culture samples are saponified to release the fatty acids. The fatty acids are then converted to their methyl esters and extracted, before being washed and separated by Gas Chromatograpy (GC). Once analysed by GC, the FAME profiles for samples are compared to those contained in a library and bacterial identification is based on the profile matches between the library and sample profile from the bacteria analysed.

Whole mouth rinses were taken as described in Section 2.2, following which, 100 µl samples were cultured under the strict conditions prescribed by MIDI Inc., USA (see Section 2.4). Representatives of each colony morphology were selected and re-streaked to purity. Samples of the pure cultures (approximately 40 mg each) were then harvested and subjected to FAME extraction as described above.

The results in Table 3.4 overleaf were obtained after culturing whole mouth rinses initially on horse blood agar (HBA), with incubation at 37 °C. Seven different colony morphologies were obtained and each type was re-streaked to purity, therefore, these FAME profiles are from representatives of common colony types cultured under the clinical conditions. Each culture was then cultured on TSA + 5 % sheep blood at 35 °C for 24 hours, before FAME profiling, according to the MIDI Inc. clinical sample preparation instructions (MIDI Inc., USA). The FAME profiles were compared to the clinical FAME library (Clin6) after GC analysis. In total, 7

samples were analysed, with 3 of those being identified as species from the genus *Rothia* and the other 4 samples corresponding to the genera listed in Table 3.4. When compared to the genera identified from RFLP analysis of whole mouth rinses, it can be seen that the genera of *Staphylococcus*, *Rothia* and *Micrococcus* are identified by FAME profiling, but not by RFLP analysis. Possible reasons for this are discussed later in this chapter.

Table 3.4.

Genus	n	Genus description
Staphylococcus	1	Firmicutes, spherical colonies form in clusters, facultatively anaerobic.
Rothia	3	Actinobacteria, aerobic/facultatively anaerobic rod, colonises oral cavity, rarely causing disease, found most often in dental plaque.
Streptococcus	1	firmicutes, facultatively anaerobic, non-motile cocci, colonise the mouth and respiratory tract, can cause infections (tonsilitis).
Neisseria	1	Proteobacteria, aerobic diplococci, generally non-pathogenic commensal microbes.
Micrococcus	1	Actinobacteria, aerobic cocci, rarely cause disease, found in many environments, can generally reduce nitrate.

Table 3.4. This table shows the results of fatty acid profiling (FAME profiling) of a whole mouth rinse. FAME profiling was carried out from cultures grown on clinical media (TSA + 5 % sheep blood) and profiles compared to the Clin6 FAME library. The genera *Staphylococcus*, *Rothia* and *Micrococcus* can be identified from FAME profiling, but are not seen in RFLP analyses of whole mouth rinses.

Table 3.5 shows the results of FAME profiling and 16S rDNA sequence analysis of a whole mouth rinse cultured under anaerobic conditions. Whole mouth rinse samples were cultured on BHIBLA plates under anaerobic conditions at 37 °C, before representatives of each colony morphology were re-streaked to purity and incubated at 35 °C for 24 hours before FAME profiling, according to the MIDI Inc. anaerobic sample preparation instructions (MIDI Inc., USA). Samples were compared to the MIDI anaerobic FAME library after GC analysis. Of the 6 samples profiled, only 1 gave a genus level identification - *Actinomyces*. To confirm the FAME profiling result and to try to identify the remaining anaerobic isolates, 16S rRNA gene sequencing was carried out from the same cultures.

16S rDNA sequencing of the successful FAME sample showed *Actinomyces* to be the most similar known genus, but only with an 83% sequence match. One other sample was identified at genus level as *Veillonella* (95% match) and 2 further samples were identified at species level as *Granulicatella adiacens* (both 99% matches). The remaining 2 samples could not be identified by DNA sequencing, as they both produced a mixed sequence result.

Table 3.5.

Genus	n	Genus description
Actinomyces	1	Actinobacteria, anaerobic/facultatively anaerobic, opportunistic pathogens of oral cavity.
Veillonella	1	Firmicutes, anaerobic, cocci, colonise the mouth and respiratory tract.
Granulicatella	2	Firmicutes, Alpha-haemolytic, related to *Streptococcus*, formerly known as *Abiotrophia*, facultatively anaerobic.

Table 3.5. This table shows the results of FAME profiling and 16S rDNA sequencing carried out on a whole mouth rinse cultured under anaerobic conditions. Only 1 genus, *Actinomyces*, was identified from FAME profiling, the other genera were identified by 16S rRNA gene sequencing from the remaining anaerobic isolates.

3.4 Prevalent genera identified by FAME dominate over time.

As the culturing of whole mouth rinses for FAME profiling resulted in only a few distinct colony types being obtained, mouth rinses were cultured and all colony types FAME profiled over a period of three consecutive days, to assess whether the few genera seen previously (see Table 3.4) were dominant and stable over time.

When cultured on both clinical media and standard nutrient agar, mouth rinses resulted in typically hundreds of colonies, with 6 or 7 distinct colony morphologies present. To ensure that these colony types were not preventing less prevalent bacteria from growing, serial dilutions of the mouth rinses were also cultured, which resulted in fewer colonies, but the same morphologies were present. In order not to presume all colony types that looked the same were indeed the same genera, representatives of each colony type from each plate were re-streaked to purity and FAME profiled. Cultures were plated on HBA plates and incubated at 35 °C for 24 hours before FAME profiling, according to the MIDI Inc. clinical sample preparation instructions. FAME profiling was carried out as described in Section 2.4.

The results in Table 3.6 overleaf show a prevalence of the genera *Micrococcus*, *Neisseria* and *Rothia* in whole mouth rinse cultures grown on the clinical media, trypticase soy agar supplemented with 5 % sheep blood (TSA-SB, Becton, Dickinson & Co.) for FAME profiling. Whole mouth rinses were taken at the same time each day, for three consecutive days, cultured on HBA and distinct single colones transferred to individual TSA-SB plates and identified by FAME profiling. Sixteen distinct colony types were observed and transferred to TSA-SB for culture. All sixteen samples were analysed by FAME profiling, resulting in the twelve genus identities shown in Table 3.6. Four of the analysed samples did not give conclusive genus identities and are therefore excluded from these data. However, each colony type was observed in abundance in the initial mouth rinse

culture, with representatives of each colony type selected for further culture and FAME profiling.

All genera have been identified previously by FAME profiling, with *Neisseria* and *Streptococcus* being identified regularly by RFLP analysis, showing that these are prevalent genera of the oral microbial flora and can be identified consistently over time.

The prevalent genera in this set of experiments are *Neisseria*, *Rothia* and *Micrococcus*. They were identified in all FAME profiling experiments over the 3 days, showing them to be both dominant and stable members of the oral flora. The ability to culture these microbes reliably and consistently over time indicates that these genera may be dominantly present in high numbers within the oral cavity.

Table 3.6.

Genus	n	Genus description
Rothia	3	Actinobacteria, aerobic/facultatively anaerobic rod, colonises oral cavity, rarely causing disease, found most often in dental plaque.
Streptococcus	1	firmicutes, facultatively anaerobic, non-motile cocci, colonise the mouth and respiratory tract, can cause infections (tonsilitis).
Neisseria	5	Proteobacteria, aerobic diplococci, generally non-pathogenic commensal microbes.
Micrococcus	3	Actinobacteria, aerobic cocci, rarely cause disease, found in many environments, can generally reduce nitrate.

Table 3.6. The results of FAME profiling of all colony types resulting from a whole mouth rinse cultured on clinical media, with samples taken at the same time each day for 3 consecutive days. *Neisseria* dominated, having being identified 5 times, with *Rothia* and *Micrococcus* also being identified on more than one occasion.

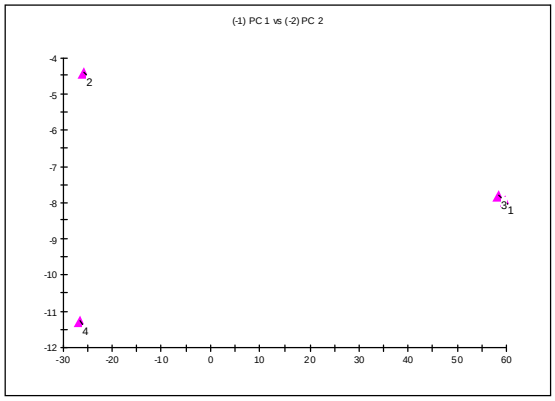
In order to present this data graphically, Principal Component Analysis (PCA) was used to produce Principal Component Plots (PC Plots), via the MIDI Sherlock Software (MIDI Inc., USA) of the diversity observed during these experiments. Briefly, PCA functions to reduce variables obtained from an experiment and turn them into artificial variables (principal components) that can be used to make predictions from the resulting data set. In this study, the 'variables' are the genera of bacteria identified in each experiment, with one 'experiment' being a single sample analysed by FAME profiling i.e. there will be several 'experiments' and many 'variables' for each complete FAME profiling analysis carried out as each FAME profiling analysis consists of multiple samples being profiled.

In the context of these experiments, genera of bacteria that are frequently identified in experiments (i.e. are common occurrences) will have low weightings, whereas those genera that are only identified infrequently (i.e. have variable occurrence) will be have a high weighting. This is Principal Component 1 (PC1). Once PC1 is completed, the analysis works again to weight the remaining variation between the samples. This is Principal Component 2 (PC2). PC1 and PC2 can be plotted against each other graphically to produce PC plots, showing the relatedness of the experiments i.e. if two experiments are very similar in terms of their results (in this case, the genera of bacteria identified), they will appear very close together on a PC Plot. If the results from two experiments are very different (e.g. one experiment identified *Streptococcus*, whereas the other identifies *Stapyhlococcus*), they will appear very far apart on a PC Plot.

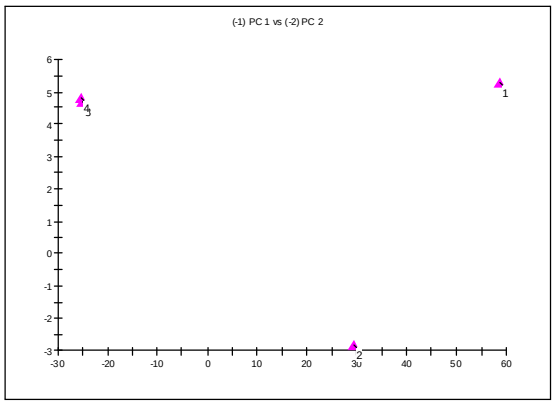
Figure 3.2 overleaf shows PC Plots of bacterial genera identified from FAME profiling over three consecutive days (results shown in Table 3.6). It can be seen that on days 2 and 3, the PC plots are identical, indicating that the outcome of each experiment was the same. This confirms that the same diversity resulted from different samples taken on completely different days. Day 1 of the analysis shows a larger number of genera being identified, hence more points are present on the PC plot. The clusters of points are far apart on the PC Plot, indicating that the genera

identified were not related. The points in each cluster are very close together, indicating that several samples within the FAME profiling experiment resulted in identifying genera that were identical or very similar to each other.

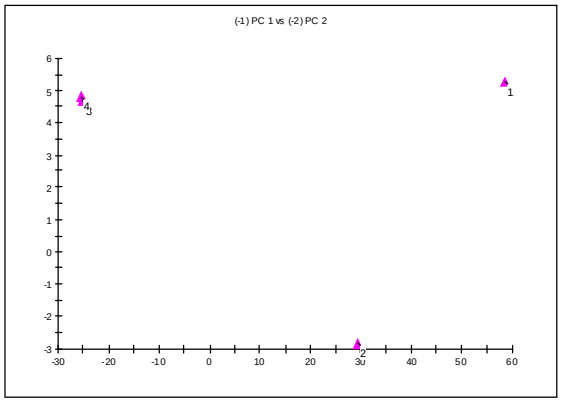
Figure 3.2.



Day 1 of 3



Day 2 of 3



Day 3 of 3

Figure 3.2. Figure showing Principal Component Plots of FAME profiling experiments performed on three consecutive days. The combined results can be seen in Table 3.6. It can be seen that on two out of the three days sampled, the diversity of the oral flora was identical, showing evidence for a stable and dominant flora over a period of time.

3.5 FAME profiling has identified many genera from mouth rinse cultures.

In order to view the entire data set gained from FAME profiling experiments and to gain insight into the amount and variability of the oral microbial diversity identified, each individual FAME profiling experiment (each bacterial sample analysed) was plotted on a PC plot. The results of this can be viewed in Figure 3.3 overleaf.

Several distinct clusters can be seen on the PC plot in Figure 3.3, indicating that there were several groups of very closely related genera identified overall. The presence of multiple points in each cluster indicates that the particular genus pertaining to that cluster was identified from multiple bacterial samples (the number of times that genus / a very similar genus was identified being equal to the number of points in the cluster). The presence of a few individual points outside of these clusters (points 6, 24, 26 and 32) indicate that these results pertain to genera that were only identified once and were not similar to those that were frequently identified and resulted in the cluster formations.

Overall, this PC plot shows that closely related genera were identified multiple times, due to the presence of many points in tight, distinct clusters. This shows stable oral microbial diversity over the long period of time (several months over one year) the multiple FAME profiling analyses were performed.

Figure 3.3.

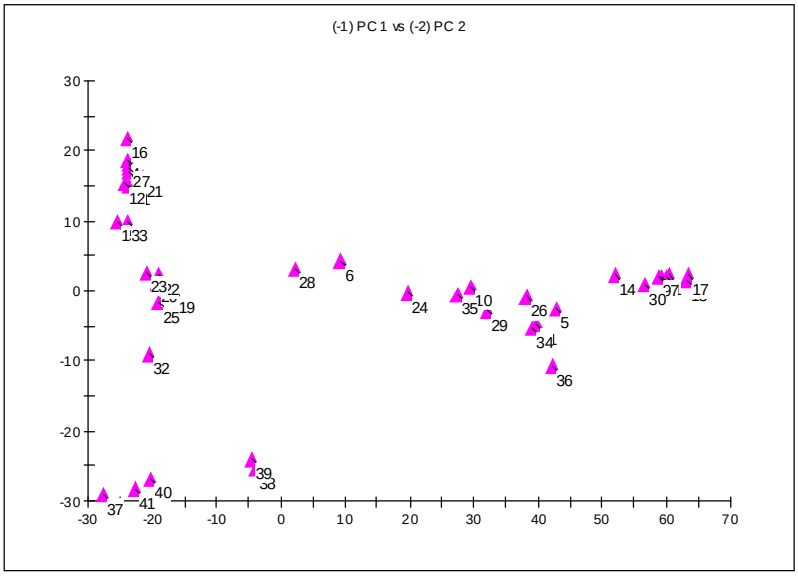


Figure 3.3. PC plot to show all results from bacterial FAME profiling experiments of pure mouth rinse cultures. Each point on the plot represents a single bacterial sample that was identified by FAME profiling. Several distinct clusters can be seen, indicating that there were several groups of closely related genera identified overall. The presence of many points in each cluster indicates that the particular genus pertaining to that cluster was identified from many bacterial samples. The presence of a few single points outside of these clusters (points 6, 24, 26 and 32) indicate genera that were only identified once.

3.6 Comparison of FAME profiling with RFLP.

In this part of the investigation, FAME profiling was being performed on pure cultures of bacteria, therefore only those organisms which could be cultured were being identified. In contrast to this, 16S rDNA sequencing can identify both organisms which can be cultured, and those that cannot. Because of this, FAME tends to identify fewer genera per analysis.

However, FAME profiling is an accurate technique for bacterial identification, as when the cultures used for FAME profiling were identified using colony PCR and 16S rRNA, the genus level identification was 100% accurate (see Tables 3.1 – 3.5). It is therefore reasonable to assume that these methods can both be used simultaneously to assess bacterial diversity from the mouth and can assess diversity both from whole mouth rinses and pure cultures taken from those rinses.

3.7 Identification of bacteria from selected lettuces and pre-packed salad products.

In order to monitor changes in bacterial diversity of the mouth following eating, it was necessary to investigate the diversity that exists on the salad produce available in supermarkets, again using both RFLP analysis and FAME profiling. It was expected that a range of microbes would be identified, ranging from soil-dwelling bacteria (from the growth of the salad produce), to microbes found commonly on the skin (transferred when handling). Swabs of the salad leaves were taken, but this resulted in poor bacterial identification (see Table 3.7 overleaf), therefore, the method was amended and salad wash samples were analysed, which resulted in many more bacterial genera being identified.

Initially, the salad leaves were sampled by rubbing a cotton swab over their surfaces for approximately 30 seconds, taking care to ensure a large area of the leaves had been sampled. The swab was then placed into the RFLP lysis buffer and agitated to release the cells. Cells were then lysed and RFLP analysis carried out as described in Section 2.3. After this method yielded poor results, the method employed by plant diagnosticians at FERA was adopted, in order to sample the bacteria present on the salad leaves by washing the leaves before cell lysis. Salad samples (10 g each) were placed in solutions of sterile 1 x PBS and TWEEN 20 and were rotated for 2 hours at room temperature, as described in Section 2.2, releasing the bacteria from the surface of the salad leaves without removing the plant cells of the salad samples. 100 µl samples of the salad washes were then lysed and RFLP analysis carried out as described in Section 2.3.

The results in Table 3.7 were obtained from an RFLP analysis of a lettuce swab taken directly from iceberg lettuces leaves. In total, 5 genera were identified, but only 3 of those were bacterial. The bacterial genera identified were *Leuconostoc, Pseudomonas* and *Erwinia* which are all commonly found on the surface of plants (Palleroni,1984, Mundt, 1970, Burkholder, 1957). However, out of the 11 clones that gave sequence data, *Erwinia* and *Leuconosto*c were only represented by 1 clone each and *Pseudomonas* was only represented by 2 clones. The genera *Lactuca* and *Lycopersicon* are plant genera (lettuce and tomato, respectively) and the BlastN DNA sequence matches for these genera were identified as chloroplast DNA. *Lycopersicon* was represented by 1 clone, but the analysis was dominated by the phylotype corresponding to the genus *Lactuca*, with 6 out of the 11 clones analysed, indicating that the plant cells from the lettuce had been sampled more than the bacteria living on the surface. This is not surprising given that the lettuce leaves were sampled by swabbing - an abrasive action that will have removed cells from the surface of the lettuce.

Table 3.7.

Genus	n	No. distinct phylotypes	Genus description
Lactuca	6	1	Common garden lettuce, distributed worldwide, some non-edible species exist.
Leuconostoc	1	1	Firmicute, heterofermentative, ovoid cocci, often form chains and are generally slime forming.
Lycopersicon	1	1	Includes tomatoes and some species of nightshade
Pseudomonas	2	1	Proteobacteria, rod shaped, strict aerobes, but certain species facultatively anaerobic (*P. aeruginosa*), can form biofilms.
Erwinia	1	1	Proteobacteria, aerobic, related to *E. coli*, member of Enterobacteriaceae.

Table 3.7. Results of an RFLP analysis of a swab taken directly from salad leaves. 3 bacterial genera were identified, along with 2 plant genera. However, the DNA sequence matches from the plant genera were identified as chloroplast DNA. As a result of these data being obtained, the method for sampling the salad leaves was modified to a wash technique, currently employed by plant diagnosticians at FERA.

Table 3.8 shows FAME profiling results of lettuce washes, carried out on four separate lettuce samples, including an organic Romaine lettuce. Each profiling result was obtained on a different day and samples were all prepared according to the diagnostic protocol from FERA. Salad samples were washed as described in Section 2.2 before 100 µl samples of the wash were cultured on nutrient agar plates and representatives of each colony morphology re-streaked to purity. Pure cultures were then transferred to TSBA plates and incubated at 28 °C for 48 hours before FAME profiling, according to the MIDI plant diagnostic sample preparation instructions. Samples (40 mg) were harvested and FAME profiled as described in Section 2.4. FAME profiles were compared to those in the TSBA6 library (MIDI Inc., USA) and the NCPPB3 library (FERA, Sand Hutton, York).

Sixteen different genera were identified and all were bacterial, showing that the lettuce wash method is much more effective than swabbing. The genera most frequently identified were *Pantoea, Acinetobacter, Stenotrophomonas* and *Staphylococcus*. The genus *Erwinia* was

represented twice, whereas *Micrococcus*, *Microbacterium* and *Bacillus* were all represented once in the analysis.

It is not surprising to have identified plant-dwelling bacteria such as *Pantoea* and *Erwinia*, as well as soil-dwelling genera such as *Acinetobacter* and *Stenotrophomonas*, as these genera are present where the lettuce grows and is harvested. During harvesting of the crop, the lettuce will be handled several times, so it is therefore unsurprising that widespread bacterial genera such as *Microbacterium* and *Staphylococcus* were identified, as these will have been transmitted to the lettuce surfaces during handling and transportation of the crop.

The organic Romaine lettuce was the fourth sample to be profiled (see Table 3.8, column n4 for results) and was specifically sampled to assess whether a greater diversity of bacterial genera could be identified from a crop which had not been subjected to pesticides or herbicides. It was hypothesised that the greatest diversity of microbes would be identified from this sample, as the soil and lettuce would be richer in microbial diversity due to the lack of man-made chemical applications. It is therefore not surprising to observe that the greatest diversity of genera was identified from this organic sample, agreeing with the hypothesis set.

Table 3.8.

Genus	n_1	n_2	n_3	n_4	Total n	Genus description
Erwinia	2	0	0	0	2	Proteobacteria, aerobic, member of Enterobacteriaceae.
Pantoea	3	1	1	1	5	Proteobacteria, aerobic, Enterobacter, widespread on plants.
Micrococcus	1	0	0	1	2	Actinobacteria, cocci, found in many environments.
Microbacterium	1	0	1	0	2	Actinobacteria, aerobic rod, heat resistant.
Bacillus	1	0	0	0	1	Firmicutes, rods, spore forming, aerobic and facultatively anaerobic, can colonise a variety of environments, including soil and humans.
Staphylococcus	3	1	0	0	4	Firmicutes, spherical colonies form in clusters, facultatively anaerobic.
Pseudomonas	0	1	0	0	1	Proteobacteia, rod shaped, strict aerobes, but certain species facultatively anaerobic (*P. aeruginosa*), can form biofilms.
Serratia	0	1	0	0	1	Proteobacteria, facultatively anaerobic, Enterobacter
Neisseria	0	1	0	0	1	Proteobacteria, aerobic diplococci, generally non-pathogenic commensal microbes.
Bordetella	0	2	0	0	2	Proteobacteria, coccobacillus, aerobic.
Acinetobacter	0	0	3	2	5	Proteobacteria, aerobic, non-motile, soil bacteria.
Curtobacterium	0	0	0	1	1	Actinobacteria, aerobic, soil bacteria, some species are plant pathogenic.
Stenotrophomonas	0	0	1	4	5	Proteobacteria, aerobic, species range from soil dwellers to opportunistic Human pathogens.
Arthrobacter	0	0	0	1	1	Actinobacteria, obligate aerobes, rods during exponential growth and cocci during stationary phase.
Kocuria	0	0	0	1	1	Previously classified as *Micrococcus*, commonly found on Human skin.

Table 3.8. Results from fatty acid profiling (FAME) analysis of lettuce washes on 4 separate lettuce samples, including an organic romaine lettuce. The samples were profiled on 4 separate occasions. More genera have been identified from this analysis and more importantly, all of the genera are bacterial, showing that this method of sampling is more effective. There is a prevalence of the plant-dwelling genera *Pantoea*, as well as the soil-dwelling microbes *Acinetobacter* and *Stenotrophomonas*.

3.8 FAME profiling has also identified many genera from salad wash cultures.

In order to view the entire data set gained from FAME profiling experiments performed on salad wash culture samples, and to gain insight into the amount and variability of the oral microbial diversity identified in the same way as was performed for the FAME profiling results of the mouth rinse cultures, each individual FAME profiling experiment (each bacterial sample analysed) was plotted on a PC plot. The results of this can be viewed in Figure 3.4 overleaf.

Similarly to the results obtained from the mouth rinse FAME profiling experiments, several distinct clusters can also be seen on the PC plot in Figure 3.4, indicating that there were several groups of very closely related genera identified overall. Again, the presence of multiple points in each cluster indicates that the particular genus pertaining to that cluster was identified from multiple bacterial samples. The presence of a few individual points outside of these clusters (points 12, 14, 26, 32 and 33) indicate that these results pertain to genera that were only identified once and were not similar to those frequently identified.

Overall, this PC plot shows that closely related genera were identified multiple times, due to the presence of many points in distinct clusters. This PC plot also shows stable microbial diversity of the salad samples analysed over a long period of time (several months over one year).

Figure 3.4.

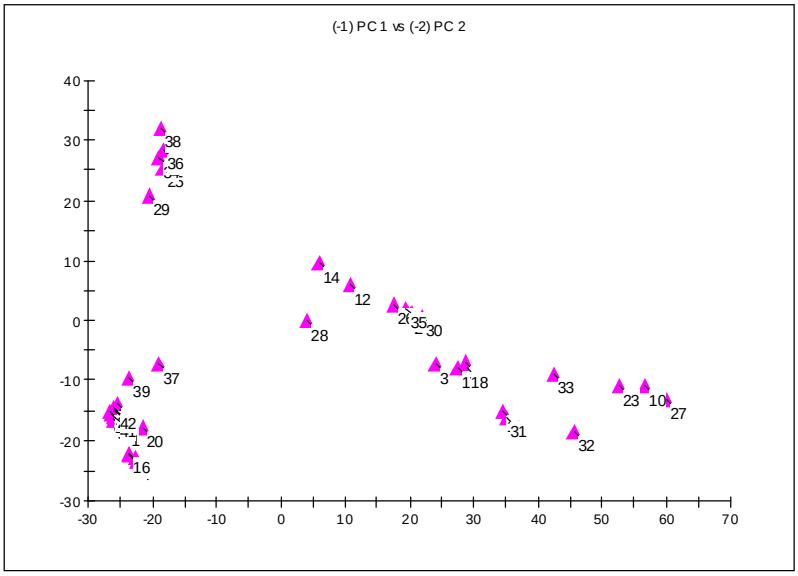


Figure 3.4. PC plot to show all results from bacterial FAME profiling experiments of pure salad wash cultures. Each point on the plot represents a single bacterial sample that was identified by FAME profiling. Several distinct clusters can be seen, indicating that there were several groups of closely related genera identified overall. The presence of many points in each cluster indicates that the particular genus pertaining to that cluster was identified from many bacterial samples. The presence of a few single points outside of these clusters (points 12, 14, 26, 32 and 33) indicate genera that were only identified once.

3.9 Direct detection of bacteria in the mouth.

To eliminate the need for culturing bacteria prior to FAME, a protocol was obtained for direct detection of bacterial FAMEs from oral rinses. This method has been used previously to identify bacteria in soil samples (Schutter and Dick, 2000), therefore, it was decided that soil samples would be used as positive controls.

Mouth rinse samples, 3.3 ml, were prepared in triplicate, alongside 3.0 g soil samples as controls. Sample preparation took place as described in Section 2.4, with the following adjustments; double volumes of reagents 1 & 2 were used (2.0 ml and 4.0 ml, respectively), samples were centrifuged at 2500 rpm for 10 minutes after the 80 °C incubation and the end over end rotation period was increased from 10 to 20 minutes, to ensure a more complete extraction and enhanced extraction of low level FAMEs. FAMEs were then analysed by gas chromatography and resulting FAME profiles compared to those stored in the TSBA6 library (MIDI Inc.).

From the literature (Cavigelli et al., 1995, Ritchie et al., 2000, Kirk et al., 2004), it is reasonable to presume that many bacterial FAMEs will be identified from the soil samples used as positive controls in these experiments. However, there are no previous studies using direct FAME detection to identify oral bacteria, so there is an uncertainty as to whether there will be enough microbial material in a mouth rinse to produce fatty acid profiles and successfully identify bacterial species.

As the FAME profiling samples are not cultured prior to analysis, they are analysed as a mixed sample. It is not possible for the MIDI software to identify bacterial genera from the samples, as the software relies on a 100% pure sample in order to compare a single FAME profile against those of the MIDI library. Because of this, the results obtained are simply a list of bacterial FAMEs extracted and these can be assigned to different types of bacteria by their type and composition. This means it is most

appropriate to view the results of such experiments graphically, as PC plots.

Representative raw data from direct detection FAME profiling experiments is shown in Table 3.5a overleaf. This figure shows the profile of bacterial FAMEs obtained from direct detection analyses and it can be seen there are many FAMEs extracted, representing many different species. The PC plot in Figure 3.5b overleaf shows the results of all FAME profiling experiments carried out from mixed mouth rinses, with no prior culturing. Each point on the plot represents a principal component, derived from each sample that was profiled.

There is an obvious clustering at -5, -3 indicating that the FAMEs identified from these samples are very similar and represent closely related results, and therefore, closely related diversity within the samples. There are also some samples (points 13, 14 and 15) that have resulted in FAME extractions that are distinct from the other samples. These FAMEs will belong to bacterial genera that are not closely related to those that form the clusters of points. This provides support to earlier FAME experiments carried out on pure cultures from mouth rinses, as the results from direct FAME detection also show stable diversity over time, with only a few experiments that resulted in FAME identification that was very different to the majority of samples analysed.

This is a very positive development in the methodologies that can be used to identify oral bacteria. Direct FAME detection has never been used to identify FAMEs and possible bacterial genera present in the oral cavity and it has been shown that this technique can be used as a method for extracting and identifying FAMEs from whole mixed mouth rinses, eliminating the need for the time-consuming prior culturing. Moreover, it has shown that the results obtained from direct FAME detection are comparable and consistent with those obtained from the analysis of pure cultures, meaning that this is a viable and reliable method for use in oral microbial diversity studies.

Table 3.5a.

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.682	2.859E+8	0.024	----	7.023	SOLVENT PEAK	----	< min rt	
1.875	186	0.019	----	7.398		----	< min rt	
2.055	346	0.021	----	7.748		----	< min rt	
2.119	403	0.020	----	7.874		----	< min rt	
2.278	228	0.022	----	8.181		----	< min rt	
3.214	653	0.028	1.061	10.000	10:0	0.41	ECL deviates 0.000	Reference 0.003
3.643	2841	0.026	1.031	10.607	11:0 iso	1.72	ECL deviates 0.001	Reference 0.004
4.669	1207	0.031	0.983	11.800	unknown 11.799	----	ECL deviates 0.001	
4.962	1428	0.034	0.973	12.091	11:0 iso 3OH	0.81	ECL deviates 0.002	
5.572	544	0.027	0.958	12.614	13:0 iso	0.31	ECL deviates 0.000	Reference 0.000
5.672	254	0.026	0.956	12.700	13:0 anteiso	0.14	ECL deviates -0.002	Reference -0.002
6.162	311	0.046	0.947	13.100	12:0 iso 3OH	0.17	ECL deviates 0.002	
6.655	1700	0.043	0.941	13.456	12:0 3OH	0.94	ECL deviates 0.002	
6.881	1730	0.035	0.938	13.619	14:0 iso	0.95	ECL deviates 0.000	Reference 0.000
7.409	5029	0.039	0.933	14.000	14:0	2.75	ECL deviates 0.000	Reference 0.000
7.584	1641	0.039	0.932	14.112	13:0 iso 3OH	0.90	ECL deviates 0.003	
8.057	1729	0.043	0.930	14.417	15:1 iso F	0.94	ECL deviates 0.002	
8.381	41033	0.041	0.929	14.624	15:0 iso	22.36	ECL deviates 0.001	Reference 0.001
8.521	49006	0.040	0.929	14.714	15:0 anteiso	26.70	ECL deviates 0.001	Reference 0.001
8.969	751	0.039	0.929	15.002	15:0	----	ECL deviates 0.002	
10.020	6621	0.043	0.933	15.627	16:0 iso	3.62	ECL deviates 0.000	Reference 0.000
10.269	4171	0.042	0.935	15.775	16:1 w9c	2.29	ECL deviates 0.001	
10.342	12005	0.045	0.935	15.818	Sum In Feature 3	6.58	ECL deviates -0.004	16:1 w7c/16:1 w6c
10.646	12205	0.043	0.937	15.999	16:0	6.71	ECL deviates -0.001	Reference -0.001
11.377	4728	0.044	0.945	16.419	Sum In Feature 9	2.62	ECL deviates 0.003	17:1 iso w9c
11.747	2774	0.045	0.949	16.630	17:0 iso	1.54	ECL deviates 0.000	Reference 0.000
11.908	18290	0.046	0.951	16.723	17:0 anteiso	10.20	ECL deviates 0.000	Reference 0.000
13.671	3655	0.047	0.982	17.721	Sum In Feature 5	2.10	ECL deviates 0.001	18:2 w6,9c/18:0 ante
13.759	1891	0.045	0.984	17.770	18:1 w9c	1.09	ECL deviates 0.001	
13.857	7171	0.056	0.986	17.826	Sum In Feature 8	4.14	ECL deviates 0.003	18:1 w7c
----	12005	---	----	----	Summed Feature 3	6.58	16:1 w7c/16:1 w6c	16:1 w6c/16:1 w7c
----	3655	---	----	----	Summed Feature 5	2.10	18:2 w6,9c/18:0 ante	18:0 ante/18:2 w6,9c
----	7171	---	----	----	Summed Feature 8	4.14	18:1 w7c	18:1 w6c
----	4728	---	----	----	Summed Feature 9	2.62	17:1 iso w9c	16:0 10-methyl

Table 3.5a. Sample raw data from direct detection FAME profiling experiments from whole mouth rinses. The FAMEs detected are listed, but are difficult to interpret due to this being a mixed sample containing many species. It is therefore more appropriate to present the data as PC plots.

Figure 3.5b.

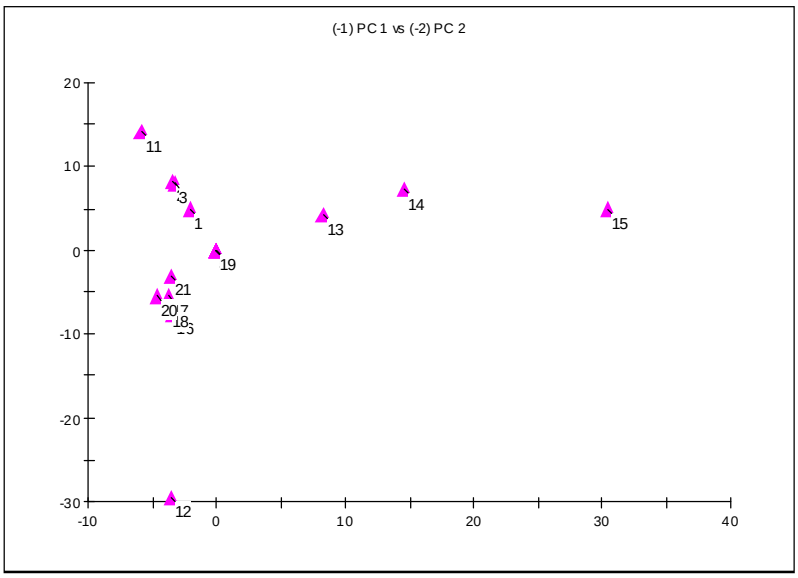


Figure 3.5b. PC plot to show all results from direct FAME detection experiments from whole mixed mouth rinses, without prior culturing. Each point on the plot represents a single bacterial sample that resulted in FAME identification (i.e. each point represents a list of bacterial FAMEs that could be associated with several bacterial genera). Distinct clustering can be seen at -5, -3, indicating several samples resulted in very similar FAME identification, thus will have very similar diversity. The presence of a few single points outside of these clusters (points 13, 14 and 15) indicate three samples that contained FAMEs that were distinct from those samples that are in the clusters.

The PC plot in Figure 3.6 overleaf shows the results of all FAME profiling experiments carried out from positive control soil samples, again with no prior culturing. Each point on the plot represents a single sample analysed i.e. a component value derived from the FAMEs extracted from each sample that was profiled.

There are three distinct clusters, which are comprised of several points each. The points in each cluster are more spread than in previous experiments, indicating that the results of these experiments are related, but not as closely related as the results obtained in previous experiments. This indicates a greater diversity of bacteria within soil samples, which is unsurprising, as previous studies have identified many different types of bacterial genera within soil samples (Cavigelli et al., 1995, Ritchie et al., 2000, Kirk et al., 2004). There are two samples (points 2 and 11) that have resulted in FAME extractions that are distinct from the other samples, as these points appear on their own and far apart from the clusters. These FAMEs will belong to bacterial genera that are not closely related to those that form the clusters of points.

The results also show that the use of soil samples as positive control samples confirms that direct detection of bacterial FAMEs can be achieved from both soil samples and whole mixed mouth rinse samples, following previously used methods (Schutter and Dick, 2000). Diversity information can be gained from both sample types and there is confidence that the results obtained from whole mouth rinse samples are valid, as results were also obtained from soil samples analysed on the same day.

Figure 3.6.

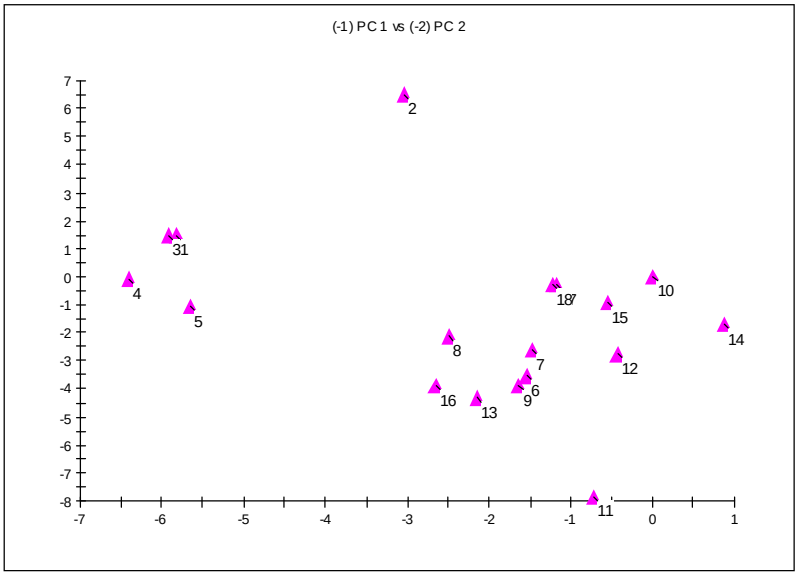


Figure 3.6. PC plot showing results of all FAME profiling experiments carried out from positive control soil samples, again with no prior culturing. There are three distinct clusters, which are comprised of several points each. The points in each cluster are more spread than in previous experiments, indicating that the results of these experiments are related, but not as closely related as the results obtained in previous experiments. There are two samples (points 2 and 11) that have resulted in FAME extractions that are distinct from the other samples, as these points appear on their own and far apart from the clusters.

3.10 Comparison of direct FAME detection with RFLP.

RFLP has been successful at identifying bacterial species without the need for culturing. However, it has also been shown that direct detection of FAMEs from mouth rinses prior to culturing is a valid method, able to provide an insight into the diversity of bacteria in whole mouth rinses without the need for culturing.

Direct detection of FAMEs is a quicker methodology, as the rinses do not need to be subjected to the lengthy period of lysis, PCR amplifications, cloning, transformations and sequencing reactions. This experimental sequence can take a few days or weeks, whereas the whole FAME extraction process and GC analysis can be performed in less than one day. This means that it can be used as a higher throughput methodology and provide many results in a short time.

However, direct detection of FAMEs can not provide identification of bacterial species in the same way as 16S rDNA sequencing, therefore, is not as informative as RFLP analysis can be. Direct FAME detection can give valuable information on the genera of bacteria present, as the different FAMEs detected and combinations thereof can be assigned to specific bacterial genera e.g. specific combinations of hydroxy fatty acids can identify gram negative bacteria, whereas gram positive bacteria lack these kinds of fatty acids and instead contain many branched fatty acids.

It can therefore be reasoned that direct extraction of bacterial FAMEs from whole mouth rinses prior to culturing is a robust method that can be used to monitor bacterial diversity within the mouth and can also be used to monitor changes in bacterial diversity both over an extended period of time and during the shorter period of time associated with eating a meal.

3.11 Monitoring diversity changes in the mouth before and after eating a salad meal.

As the diversity data was only collected from one individual, a longer, more detailed experiment was carried out. RFLP has so far been used successfully to give a 'snap-shot' of the diversity in the mouth, at one point in time (i.e. the point at which the mouth rinses are taken). However, monitoring diversity before and after a meal would also assess whether the RFLP identification method could be used consistently to produce lots of data over a longer time-course. This experiment may give an insight as to whether the oral microbial diversity changes significantly after eating a meal, or whether food-borne microbes are identified transiently within the mouth immediately after eating, after which they are no longer identified, with the oral diversity remaining stable.

Mouth rinse samples were taken each hour, beginning 2 hours before eating a salad meal and ending 3 hours after finishing the meal. Samples were taken immediately before and after eating the meal, as well as a sample of the meal being taken for RFLP analysis. Each mouth rinse was taken as described in Section 2.2 and analysed by RFLP as described in Section 2.3. Forty positive transformants were screened at random, per time point and the results for the experiment are shown in Table 3.9.

Table 3.9 shows the diversity within the oral cavity, assessed by RFLP, over a 5 hour time course, before and after eating a salad meal, as previously described.

It can be seen that many of the same genera were identified a number of times and at multiple time points, namely *Prevotella*, *Streptococcus* and *Neisseria* were all identified at all time points. *Rothia* was identified at +1 and +2hr time points, notably only present after eating the salad. However, *Rothia* was not identified from the salad meal, therefore it is not possible to say that this genus was present as a result of eating. Also, it is

not possible to conclude that *Rothia* was not present in the mouth before eating the salad. It is reasonable to presume *Rothia* was present in the mouth throughout the experiment, as this genus has been identified previously in earlier diversity studies on mouth rinse samples and is a known oral inhabitant. However, *Rothia* was not identified at every time point during this experiment and this could be due to the sample size not being large enough to ensure that the most prevalent and identifiable genera are being sampled.

The oral microbial diversity before and after eating a meal does not appear to vary greatly and the genera identified multiple times both before and after eating the meal are the same. However, to assess this statistically, results were analysed by a statistical diversity program called EstimateS (http://viceroy.eeb.uconn.edu/EstimateS) (data not shown). This does not give a conclusive result and this likely relates to the fact that the sample size is not large enough to have produced the greatest possible insight into the diversity present in all samples.

However, what is clearly shown by these results is that the genera identified after eating the salad meal are all oral microbes and none are those microbes identified from the salad sample. This indicates that the food-borne microbes do not remain in the mouth after eating and are washed from the mouth by the saliva during eating and exit the mouth when the food is swallowed.

Overall, this experiment showed that the diversity within the mouth appears stable over time and does not change dramatically as a result of eating a meal. This is not wholly unsurprising, as the oral flora is established early in life (Long and Swenson, 1976) and may evolve over time due to age and subsequent oral health (Kazor et al., 2003, Li et al., 2007), but will remain stable over long periods of time (Marsh, 1989). Microbes from food may be present transiently in the mouth and be washed out of the mouth with the saliva when swallowing. This would make it difficult to accurately sample these food-borne microbes, as they

would not be present in high enough numbers or be present in the mouth for long enough.

Table 3.9.

	Genus	n
Before Eating Salad Meal	Streptococcus	8
	Fusobacterium	3
	Prevotella	4
	Neisseria	2
	Veillonella	1
	Gemella	3
	Actinomyces	1
	Lachnospiraceae (oral clone)	1
	TM7 (oral clone)	1
	Unknown genus (oral clone)	1
Salad Meal	Lactuca, chloroplast	1
	Leuconostoc	1
	Lycopersicon	1
	Pseudomonas	1
	Erwinia	1
After Eating Salad Meal	Streptococcus	2
	Fusobacterium	0
	Prevotella	3
	Neisseria	2
	Veillonella	1
	Rothia	2
	Porphyromonas	1
	Unknown genus (oral clone)	1

Table 3.9. Results from an experiment to investigate the bacterial genera present in the mouth over a 5 hour time course, before and after eating a salad meal. Mouth rinses were sampled every hour, as well as immediately before and after eating the salad meal. A sample of the salad was also analysed.

Table 3.10.

Phylum	Class	Order	Family	Genus
Proteobacteria	Beta-proteobacteria	Neisseriales	Neisseriaceae	*Neisseria*
Firmicutes	Clostridia	Clostridiales	Acidaminococcaceae	*Veillonella*
Bacteroidetes	Bacteroidetes	Bacteroidales	Prevoteflaccae	*Prevotella*
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*
Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	*Eubacterium*
Eubacteria	Fusobacteria	Fusobacteriales	Fusobacteriaceae	*Fusobacterium*
Bacteroidetes	Bacteroides	Bacteroidales	Porphyromonadaceae	*Porphyromonas*
Firmicutes	Bacilli	Baccillales	Staphylococcaceae	*Gemella*
Actinobacteria	Actinomycetales	Actinomycineae	Actinomycetaceae	*Actinomyces*
Actinobacteria	Actinomycetales	Actinomycineae	Micrococcaceae	*Rothia*
Actinobacteria	Actinomycetales	Actinomycineae	Micrococcaceae	*Micrococcus*
Firmicutes	Bacilli	Bacillales	Bacilli of uncertain affinities	*Granulicatella*
Dicots	Asterids	Asterales	Asteraceae	*Lactuca*
Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	*Leuconostoc*
Dicots	Asterids	solanales	Solanaceae	*Lycopersicon*
Proteobacteria	γ-Proteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
Proteobacteria	γ-Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Erwinia*
Proteobacteria	γ-Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Pantoea*
Actinobacteria	Actinomycetales	Actinomycineae	Microbacteriaceae	*Microbacterium*
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*
Proteobacteria	γ-Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Serratia*
Proteobacteria	B-Proteobacteria	Burkholderiales	Alcaligenaceae	*Bordetella*
Proteobacteria	γ-Proteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*
Proteobacteria	γ-Proteobacteria	Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*
Proteobacteria	γ-Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Salmonella*
Actinobacteria	Actinomycetales	Actinomycineae	Micrococcaceae	*Arthrobacter*
Actinobacteria	Actinomycetales	Actinomycineae	Microbacteriaceae	*Curtobacterium*
Actinobacteria	Actinomycetales	Actinomycineae	Microbacteriaceae	*Kocuria*
Actinobacteria	Actinomycetales	Corynebacterineae	Corynebacteriaceae	*Corynebacterium*

Table 3.10. Classification of all genera identified in the study. The phyla Firmicutes, *Proteobacteria* and *Actinobacteria* are most commonly identified.

3.12 Discussion.

3.12.1 Diversity of organisms identified in the mouth and on salad samples.

There have been many studies which have assessed the diversity of microbes within the oral cavity. These studies mainly centre around sampling the periodontal pockets of the mouth (Haffajee et al., 1998, Sakamoto et al., 2005, Paster et al., 2006), although other oral sites have also been sampled (Aas et al., 2005). Aas et al. sampled nine different oral sites; dorsum of the tongue, lateral sides of the tongue, buccal fold, hard palate, soft palate, labial gingiva and tonsils of soft tissue surfaces, using sterile cotton swabs. Supragingival and subgingival plaque from tooth surfaces was also sampled using a sterile curette. It is reasonable to presume that the oral sites sampled in the present study are comparable with those sampled by Aas, with the exception of the plaque samples, as the samples for this study were taken in the form of whole mouth rinses, which were washed around the mouth and tonsils and will have captured bacteria from all sites listed in the study by Aas (Aas et al., 2005). It is also reasonable to presume, therefore, that the same genera of bacteria will have been identified in the present study as were identified in the study by Aas.

From the study performed by Aas et al. in 1995, bacteria from six different phyla were identified. These phyla included the *Firmicutes* (such as species of *Streptococcus*, *Gemella*, *Eubacterium* and *Veillonella*), the *Actinobacteria* (such as species of *Actinomyces*, *Atopobium* and *Rothia*), the *Proteobacteria* (e.g. species of *Neisseria* and *Eikenella*), the *Bacteroidetes* (e.g. species of *Porphyromonas* and *Prevotella*), the *Fusobacteria* (e.g. species of *Fusobacterium* and *Leptotrichia*), and the TM7 phylum, for which there are no cultivable representatives (Aas et al., 2005), but their involvement in oral disease has been explored (Brinig et

al., 2003). Developments in technology have resulted in more information being available on the physiology of the bacteria that make up this phylum, after a microfluidic system was developed that allowed 16S DNA sequencing from single bacterial cells (Marcy et al., 2007).

Overall, Aas et al. found that cultivable and not-yet-cultivable species of *Gemella*,
Granulicatella, *Streptococcus*, and *Veillonella* were commonly detected in most sites of the oral cavity (Aas et al., 2005). Two of these four genera were most prevalent in the present study, with *Neisseria* being the other dominant genus identified from samples in this study.

A striking difference between the study carried out by Aas et al. and the study described in this thesis is the number of 16S clones analysed in diversity studies. The study by Aas et al. in 1995 was based on the analysis of 2589 16S rRNA clones, whereas, the data in this study were based on only hundreds of analysed clones. There is clearly a huge difference in the number of clones analysed, but it is interesting to note that members of all six phyla identified by Aas et al. have been identified in the present study, with two of the four most prevalent genera being identical. It has been shown previously that the genus Neisseria is identified prominently in certain regions of the oral cavity (Aas et al., 2005), which is also a comparable result with the present study. The data presented in this thesis support those previously obtained by Aas et al. so there is confidence in the methodology and identifications.

Moreover, Aas et al. also helped to build emerging bacterial profiles that help define the healthy oral cavity. Genera such as *Streptococcus* and *Veillonella*, were detected in most or all oral sites, whereas several species were quite site specific. For example, *R. dentocariosa*, *Actinomyces* spp., *S. sanguinis*, *S. gordonii*, and *A. defectiva* appeared to preferentially colonize the teeth (Aas et al., 2005). All of these genera have been identified in diversity experiments within this thesis, so there is confidence in a healthy oral cavity having being sampled, as well as

confidence in the methodology used, as whole mouth rinse analyses identified genera that have been previously identified by sampling individual sites in the mouth (Aas et al., 2005).

Before the major study by Aas et al. in 2005, Kazor et al. performed a study in which the bacterial diversity present on the tongue dorsa of healthy subjects were analysed alongside samples from subjects suffering from halitosis (bad breath) (Kazor et al., 2003). The species identified prevalently in healthy patients (and therefore most associated with health) were *Streptococcus salivarius*, *Rothia mucilaginosa* and an uncharacterized, cultivable species of *Eubacterium* (strain FTB41) (Kazor et al., 2003). Once again, the results from the analysis of whole mouth rinses in the present study are comparable, with all genera identified in this study. This further strengthens the confidence in the methodology, as the same genera can be sampled by using the whole mouth rinse technique. The sample size for the study by Kazor et al. was approximately 750 analysed clones, with 50 – 100 clones analysed per subject (Kazor et al., 2003). In the diversity study discussed in this thesis, 40 – 50 clones were analysed during each RFLP experiment, making the results of this study and that of Kazor et al. (2003) particularly comparable.

A more recent study (Quinque et al., 2009), has examined diversity in the human salivary microbiome, a study not previously carried out. During this study, saliva samples were obtained from 120 individuals, from six geographic regions. A highly variable portion of the 16S rRNA gene of 500 bp was amplified and cloned, and approximately 120 clones were sequenced from each individual (Quinque et al., 2009). A total of 14,691 sequences were obtained, which were then analyzed for possible chimeras or other artifacts. Excluding the 576 potentially chimeric sequences, a total of 14 115 sequences were selected for further analysis. The number of genera identified in each individual ranged from six to thirty. Sixteen genera were observed only once, while the most frequently identified genus, *Streptococcus*, accounted for 22.7% of the total number of sequences analysed (Quinque et al., 2009).

Once again, the study by Quinque et al. (2009) is comparable to the diversity results presented in this thesis, in terms of the numbers and types of genera identified. The striking difference between the two studies is the sample size, with the study by Quinque et al. consisting of a huge sample number (14 115, compared to the hundreds of sequences analysed in the diversity study discussed in this thesis). However, it is worth noting that the diversity study carried out in this thesis was intended to be a systematic examination of the diversity within the oral cavity, performed in order to identify the prevalent genera for further interaction studies. It is interesting, however to observe the results of such a small study being comparable to much larger studies.

When analysing the results obtained from the microbial diversity examination of pre-packaged salad samples in this study, it was interesting to refer to the first study that provided information on the microbiological quality of organic vegetables (Sagoo et al., 2001). This study assessed the microbial load of the pathogenic bacteria *Listeria*, *Salmonella*, *Campylobacter* and *E. coli* O157 in each of the samples. The results showed that there was an absence of these pathogens in the 3200 samples analysed (Sagoo et al., 2001), which agrees with the results obtained from the salad rinse samples analysed in the present study, where no pathogenic bacteria were identified.

A more recent study that assessed the microbial quality and diversity present in pre-packaged spinach and lettuce leave salads also found the prevalence of potentially pathogenic coliforms to be low (Valentin-Bon et al., 2008). This study analysed 100 bagged lettuce and spinach samples and showed a mean bacterial count of 7.0 log10 CFU/g per sample. Generic *E. coli* bacteria were detected in 16% of samples, but cell counts were low, with most probable numbers (MPN) per gram being ≤ 10 (Valentin-Bon et al., 2008). These results again agree with the observations in the present study, that no potentially pathogenic bacteria

can be identified from salad wash samples. There is no literature employing the same method of analysis at this time.

3.12.2 Issues with sample size – Is there confidence in the results obtained?

The issues and effects of sample size on microbial diversity have been discussed specifically in the context of soil biodiversity (Ranjard et al., 2003, reviewed in Kang and Mills, 2006), and more generally (Gotelli and Colwell, 2000).

Generally, as more individuals are sampled, more species will be recorded (Bunge and Fitzpatrick, 1993, Gotelli and Colwell, 2000). The question, therefore, is how many samples are required to ensure that a complete assessment of microbial diversity has been achieved, with all the species being sampled and identified. Communities may differ in measured species richness because of differences in underlying species richness, or because of differences in the number of individuals counted or collected (Denslow 1995). Differences in abundance may reflect differences in sampling effort (Denslow 1995), therefore it is important to ensure enough samples are analysed in order to be confident of the results obtained.

Kang and Mills indicate that there is a clear beneficial effect of increasing sample size in terms of better representation of the environment from which samples are collected (Kang and Mills, 2006). This is based on the study by Ranjard et al. in 2003, where the issues of sample size in soil microbial diversity were explored (Ranjard et al., 2003). This study highlights the requirement for a satisfactory sampling strategy that takes into account the high microbial diversity and the heterogeneous distribution of microorganisms in the soil matrix, in order to gain a true representation of the soil microbial diversity (Ranjard et al., 2003). During analyses in that study, the influence of the sample size of three different soil types on the DNA yield and analysis of bacterial and fungal community structure were investigated. Variations between bacterial and fungal community structure were quantified by using principal component analysis. DNA yields were positively correlated with the sample size,

suggesting an influence of the sample size on DNA recovery (Ranjard et al., 2003). This is not entirely surprising, as it is reasonable to presume that the larger the individual sample / number of samples, the more DNA will be available for analysis. One conclusion drawn from that study was that a large number of small samples should be analysed, in order to gain a more complete inventory of microbial diversity (Ranjard et al., 2003). The diversity study discussed in this thesis follows this principle, as a number of mouth rinses were sampled, with a number of clones analysed from each rinse. This builds confidence that the methodology employed during the diversity study was correct and that the study was being performed in an appropriate manner to gain a complete assessment of microbial diversity. It is, again, worth noting that the diversity study carried out in this thesis was intended as an evaluation of microbial diversity only and was the initial phase of the overall study. However, had the diversity examination continued for the entirety of the study duration, employing the same methodology, with more clones analysed per RFLP analysis, there could be confidence that the species richness and diversity would have been assessed in a more complete way, with stronger data sets for statistical analysis achieved.

3.12.3 The methodology employed influences the bacterial genera identified.

When assessing the data obtained from RFLP and FAME profiling, clear differences can be seen in the types of genera identified from each method. Firstly, *Staphylococcus* and *Micrococcus* can be identified by using FAME profiling, but are not identified and therefore are under-represented in RFLP analyses. This may be due to the fact these genera are grm positive and therefore more difficult to lyse in the first steps of RFLP. Anaerobic bacteria, such as *Fusobacterium* and *Porphyromonas*

are identified in RFLP analyses, but not from FAME profiling experiments. Indeed, the identification of anaerobic bacteria in general is difficult when using FAME profiling, as poor FAME extraction and genus / species identification occurs when employing the anaerobic FAME profiling method from the MIDI protocol (MIDI Inc., USA). The results of the anaerobic FAME profiling carried out in this study (see Table 3.5) were verified by 16S rDNA sequencing in order to strengthen the poor quality results obtained from FAME profiling.

The under-representation of *Staphylococcus* in RFLP analyses may best be explained by the difficulty in lysing staphylococcal cells. This can often be difficult, due to the thick peptidogylcan layer present in the Staphylococcal cell structure (Bera et al., 2005). There is also the suggestion that some staphylococci are resistant to lysozyme due to the the peptidoglycan *O*-acetyltransferase OatA (Bera et al., 2005). Similarly, the genus *Rothia* is also lysis resistant (Gustafson et al., 1985) and this could mean that there was an under-representation of this genus in RFLP analyses, even though this genus was identified in the diversity study discussed in this work (see Table 3.9) and previously (Aas et al., 2005).

In contrast to *Staphylococcus* and *Rothia*, the genus *Micrococcus* is susceptible to cell lysis (D'Souza and Marolia, 1999). It is therefore surprising that this genus was not identified by RFLP analyses, but was readily and consistently identified by FAME profiling. It may be that this genus is easy to culture (as it does grow on all types of FAME profiling media), but its 16S rDNA does not amplify well, meaning that it is identified from pure FAME profiling cultures, but not from DNA amplification from whole mouth rinses.

3.12.4 The advantages and pitfalls of the methodologies employed.

The major advantage of using RFLP as a method for assessing microbial diversity is that this method can identify those microbes which cannot be readily cultured. It is also a useful method for identifying anaerobic bacteria that may be difficult to culture under standard laboratory conditions and are therefore under-represented in culture-based studies. This also removes the need for prior culturing steps and the need for complex microbiological media for isolating different types of bacteria.

Although the analysis of microbial diversity by RFLP is a powerful tool, allowing thousands of 16S rDNA sequences to be analysed and assigned to bacterial species, thus giving a picture of the bacterial diversity present in the sample(s), there are several pitfalls that must be considered and taken into account during analysis. These are discussed in the following section.

The initial step in an RFLP analysis is cell lysis and extraction of the DNA for amplification. Insufficient or preferential disruption of cells will most likely bias the view of the composition of microbial diversity as DNA, which is not released from the cells, will not contribute to the final analysis of diversity (von Witzengerode et al., 1997). Conversely, rigorous conditions required for cell lysis of Gram-positive bacteria should be avoided as this treatment may lead to highly fragmented nucleic acids from Gram negative cells, which can lead to artefact formation during PCR amplification (von Witzengerode et al., 1997)

Following cell lysis, DNA purification steps may take place, to ensure the nucleic acids are as pure as possible before amplification occurs, which can result in a loss of nucleic acids (von Witzengerode et al., 1997). For this reason, this step was not taken in this study in order to preserve the amount of DNA obtained from cell lyses.

The most crucial step of the RFLP analysis is the successful amplification of 16S rDNA fragments for analysis. In PCR amplification of 16S rDNA from complex microbiota a mixture of homologous molecules serve as template. Amplified DNA can only reflect quantitative abundance of species if the amplification efficiencies are the same for all molecules (von Witzengerode et al., 1997). To be sure the amplification efficiencies are equal, the following assumptions have to be made; all DNA molecules are equally accessible to primers, primers anneal to each DNA template with equal efficiency and the DNA polymerase efficiency is equal throughout the PCR reaction (von Witzengerode et al., 1997). These assumptions become difficult when it is known that universal primers used in such PCR amplifications can contain degeneracies that can influence the annealing of primers to DNA templates in the reaction mix (von Witzengerode et al., 1997).

One of the main problems associated with the PCR amplification step of an RFLP analysis is the presence of contamination with non-target DNA, which can enter reactions via tube-to-tube contamination, or by contaminated reagents (von Witzengerode et al., 1997). If contaminating DNA contains the primer annealing sequence, the contaminating DNA will be readily amplified and will be identified both in negative controls (with no DNA template present) and as an additional amplification product in experimental samples (von Witzengerode et al., 1997). If such contaminating DNA was subsequently cloned and sequenced for identification against nucleotide databases, an over-representation of bacterial diversity will be obtained.

Although about 5000 full and partial 16S rRNA and 16S rDNA sequences of cultivated microorganisms and environmental clones have been released this number reflects only a minor part of the expected microbial diversity (von Witzengerode et al., 1997). There is also a question mark as to whether the sequence data for bacteria contained within nucleotide databases is of high enough quality to assign species identities to bacteria

from environmental samples, where the sequence similarities are low (von Witzengerode et al., 1997).

More recently, Janda and Abbot have discussed the usefulness of 16S rRNA gene sequencing as a tool in microbial identification. In this study, Janda and Abbot make the point that microbial identification is based upon both assumptions that the nucleotide sequences in databases are correct and that they have been correctly assigned to the species identified (Janda and Abbot, 2007). There is some thought that there may be some errors associated with sequences, with 5% of studied sequences containing some error (Ashelford et al., 2005, Janda and Abbot, 2007).

Moreover, when assigning species identifications to sequences obtained from RFLP analyses, no universal definition for species identification via 16S rRNA gene sequencing exists, and authors vary widely in their use of acceptable criteria for establishing a "species" match (Janda and Abbot, 2007). Species 'matching' does not exceed 99% similarity, raising the question of whether the identification levels applied are sufficient to gain an accurate identification of the species (Janda and Abbot, 2007).

The molecular biological approach to assessing bacterial diversity may be costly, with reagents and enzymes being expensive to procure. In contrast to this, FAME profiling produces a cheap and reproducible method for analysing bacterial diversity (Stead, 1995). FAME profiling is also a quick method to employ, taking a matter of hours to analyse tens of samples following the culture periods, whereas, the various steps required during RFLP analyses can take several days following initial amplification.

FAME profiling results are also highly accurate, being 98% accurate at identifying bacteria to species level (Stead, 1995). The results obtained are also comparable to those obtained by RFLP analyses, as shown in this study, where FAME profiling results were confirmed by 16S rDNA sequencing. Furthermore, slight changes in culture conditions do not affect the overall outcome of the FAME profiling (Stead, 1995), whereas,

slight changes in RFLP techniques e.g. cell lysis procedure or PCR cycling temperatures) may have a significant impact on the amount of DNA amplified for analysis and therefore affect diversity results obtained.

However, the main drawback of the FAME profiling methodology is that it cannot identify those bacteria that cannot be cultured, hence this method is missing this portion of the diversity that can be captured by using RFLP. In addition to this, anaerobic bacteria (such as *Porphyromonas* and *Fusobacterium*) will be under-represented in FAME profiling experiments, as the culture of anaerobes proves difficult under standard laboratory conditions. Furthermore, the MIDI protocol that defines the procedure for FAME profiling requires the use of very specific culture media to be used (MIDI Inc., USA), which in turn requires specific preparation and culture conditions in order to be compliant with the MIDI Sherlock bacterial identification software (MIDI Inc., USA).

On of the main difficulties faced when assessing microbial diversity by FAME profiling is the interpretation of the results, particularly when direct detection of FAMEs is performed, with no prior culturing. The profiles of acids can be difficult to decipher and require developed expertise to assign the types of FAMEs extracted to particular bacterial genera (Stead, 1995). Moreover, the interpretation of FAME profiles from environmental samples can be hard to interpret, as many microbes contain common FAMEs, leading to the gathering of only qualitative descriptions of the types of FAMEs present (Cavigelli et al., 1995). For this reason, the interpretation of the results can be a limiting factor as to the usefulness of the method, especially if access to appropriate expertise is lacking.

3.12.5 Direct detection of microbial FAMEs was successful but may require further development to be a more successful tool for assessing diversity.

The application of direct FAME detection has mainly been in assessing microbial diversity of environmental samples such as soil (Ritchie et al., 2000, Cavigelli et al., 1995), fungi (Graham et al., 1995) and compost (Carpenter-Boggs et al., 1998). As a replicable method for direct FAME extraction has been elicited (Schutter and Dick, 2000), it was reasonable to assume that some insight into the microbial diversity of the mouth could be obtained by direct FAME detection.

Indeed, this was the case, as is shown in Figure 3.5 earlier in this chapter. However, the results obtained from these experiments were not as numerous as those obtained in a previous study by Cavigelli et al. in 1995, where soil samples were analysed for diversity by direct FAME detection (Cavigelli et al., 1995). In the study by Cavigelli et al., 162 soil samples were analysed, with each sample producing a similar FAME profile consisting of, on average, 24 FAMEs (Cavigelli et al., 1995). Clearly this is a much larger number of samples when compared to those analysed in the direct FAME detection experiments discussed in this thesis, however, it is to be noted that the soil samples analysed in this study were intended as positive control samples only.

The mouth rinses that were analysed by direct FAME detection in this study produced profiles consistingof around 5 FAMEs. When compared to the positive control samples, where profiles comprised around 15 FAMEs, the level of FAME detection and extraction is moderate, providing initial confidence that the method can be employed in assessing bacterial diversity from complex mixed samples. It is unsurprising that the number of FAMEs extracted from mouth rinses is less than that extracted from soil samples, as mouth rinse samples will contain a lower concentration of

bacteria than the equivalent amount of soil, due to dilution from the sterile water used to rinse the mouth.
An interesting point to note is that more than 80% of the total number of FAMEs extracted from soil samples by Cavigelli et al. were observed in the first 12 samples analysed (Cavigelli et al., 1995), indicating that the diversity was present in only a small part of the sampling size. This observation leads to the question of whether only a small number of mouth rinses would have to be analysed by this method in order to obtain information on the majority of the bacterial diversity present.

A larger-scale and more long-term study would be required in order to investigate this more fully, but there can be confidence that direct detection of FAMEs can be used as a method for analysing bacterial diversity from whole mouth rinses, an application for this technique that has not been discussed previously in the literature to date.

3.12.6 Concluding remarks.

Overall, this chapter has given an insight into the microbial diversity present in both the oral cavity and in pre-packaged salad products that are commercially available. The experiments presented in this chapter were carried out over 18 months of the total study period and give good support to a stable microbial community. The same genera were identified consistently and by the use of different methodologies, again supporting the evidence for a stable community that persists over time. No evidence could be found for a changing microbial community, with this observation being comparable to that made previously by Marsh (1989).

However, to be more confident in the statements above, a larger-scale study is required, performed over a longer time period, in order to strengthen the data for a stable, long-term microbial community in the mouth. The increased number of samples that such a study would provide

would mean that statistics could be performed in order to give an insight into the species richness and whether the majority of the diversity present had been sampled. Nevertheless, the results already obtained are a useful start to such a study and serve as a good evaluation of the diversity present in the Human mouth and have identified prevalent genera which can be studied in greater detail.

During the assessment of bacterial diversity in the mouth over the course of eating a meal, no food-borne microbes could be identified in the mouth immediately after eating (see Table 3.9), indicating that food-borne microbes do not persist in the mouth during or after eating. This may be due to the action of saliva removing them from the mouth during swallowing, or that they are washed out of the mouth during the meal if a drink is taken with the meal. At this time, there is no literature to support these statements and a much larger-scale study is needed, including more subjects and time points, to be more certain of this observation.

4. Analysis of interactions betweenpPrevalent genera of the oral microflora during co-culture.

4.1 Investigating interactions between prevalent microbes identified in the mouth – Selection of genera.

After performing a systematic evaluation of the diversity of microbes present in the mouth, it could be seen that the most prevalent genera were *Neisseria* and *Streptococcus*, both of which were consistently identified by both methods used. Indeed one, or both of these genera were identified in each anaylsis performed on a mouth rinse. The genus *Veillonella* was also prevalent and members of this genus were identified frequently during the diversity study. The prevalence of these genera indicates that these microbes are consistently present in the mouth and may be interacting with each other. In order to investigate this further, it was necessary to perform co-culture experiments to assess whether any of the genera impacted the growth of the others. *Veillonella* is a good candidate to include in these investigations alongside *Streptococcus* and *Neisseria*, as it is classified as an anaerobe and could give valuable information into the potential interactions between anaerobes and aerobes in the mouth.

4.2 Bacterial Overlay Experiments.

The first step taken to investigate the potential interactions between the microbes was to see if there was any impact upon any of the genera when they were cultured together on and in solid media in a bacterial overlay plate experiment. This would mimic the potential interaction of the genera if they were inhabiting the same environment within the oral cavity. This methodology required development and optimisation for the system, particularly in order to ascertain the optimal inocula of bacteria and the

optimum conditions for growth of the three genera both on solid culture media and in liquid cultures. It was also important to develop a method that could be used routinely and reliably in these experiments. The methodology also had to give some qualitative information about the possible interactions e.g. being able to visualise / measure the effect of one genus inhibiting or promoting the growth of another. It would then be possible to observe trends across experimental repeats and have a good base on which to develop further experimental techniques required to investigate any interactions in more detail.

Bacterial overlay experiments were carried out to assess interactions between the dominant genera identified (*Streptococcus*, *Neisseria* and *Veillonella*), using the isolates obtained from mouth rinse cultures. All experiments were carried out in triplicate. The overlay methodology provided an opportunity to visualise any impacts on growth, either positive or negative, when the genera were grown in various combinations. A 10 µl spot of bacteria was placed in the centre of a HBA plate and allowed to grow overnight, incubated at 37 °C under the following conditions; *Nesseria* were cultured in 5 % CO_2, *Streptococcus* in 5 % CO_2 and *Veillonella* cultures were maintained under anaerobic conditions in an anaerobic gas jar (Oxoid), with the addition of an AnaeroGen sachet (Oxoid) and an anaerobic indicator strip (Oxoid). Single spots of bacteria were used, as any areas of clearing / overgrowth that may occur in the overlay could be clearly seen over and immediately around the spot.

It was necessary to determine the optimum inoculation of bacteria in the overlay, in order that the relative amount of bacteria in the overlay was not too high as to overcome any potential inhibition of growth from the bacterial spot. Initial experiments to investigate this were designed, with the inoculum of the overlay set at 1 ml bacteria in 9 ml molten 0.3 % MHA, in which the bacteria were in either mid-log phase (OD_{600nm} = 0.5 – 1.0) or were in late log / stationary phase (OD_{600nm} = 3.0 – 5.0).

Figures 4.1 and 4.2 overleaf show representative results of these experiments. The photograph in Figure 4.1 shows the result of an overlay experiment where the overlay is inoculated with a mid-log phase culture. There is some prominent growth of bacteria in the overlay, but this is probably caused by cells from the bacterial spot being incorporated into the overlay as the molten MHA is poured on to the plate. Mixing of the bacterial spot with the overlay agar was minimised in later experiments by ensuring the overlay agar was applied slowly over a plate held on a slant. In spite of the spot mixing with the overlay in the result shown in Figure 4.1, the bacterial spot can be clearly seen, along with an area of clearing over and around the spot. The overlay is not completely opaque due to bacterial growth, therefore, any regions of killing / overgrowth indicating interactions will be clearly visible.

Figure 4.2 shows the representative result of an overlay experiment in which the overlay contains a high inoculum of bacteria. There is significantly more bacterial growth in the overlay, which is not surprising, but this makes it much more difficult to see the bacterial spot and visualise any areas of clearing / overgrowth that may be caused by the bacterial spot. It was therefore decided that the lower inoculum of bacteria should be used in the overlay in all experiments.

Figure 4.1.

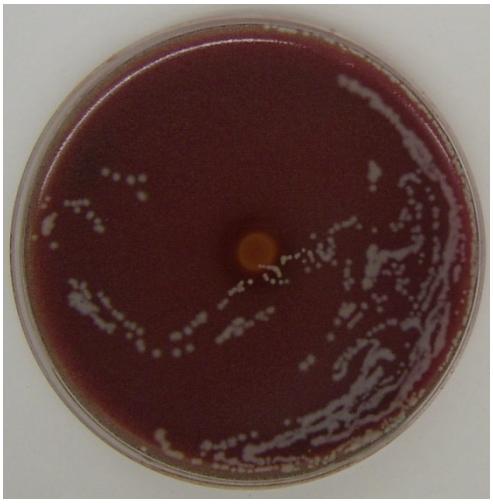


Figure 4.1. Photograph of a bacterial overlay with a low inoculum of bacteria. The bacterial spot is *Streptococcus*, with the overlay inoculated with *Neisseria* at a low $OD_{600\ nm}$ (0.5 – 1.0). Despite some overgrowth due to incorporation of the bacterial spot cells into the overlay upon pouring, the bacterial spot can be clearly seen, as can the area of clearing over and around the spot.

Figure 4.2.

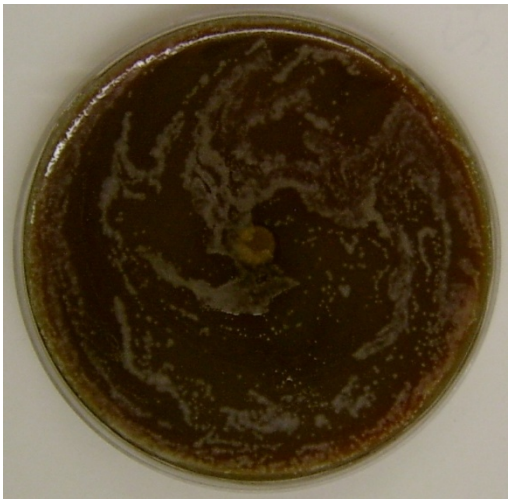


Figure 4.2. Photograph of a bacterial overlay with a high inoculum of bacteria. The bacterial spot is *Streptococcus*, with the overlay inoculated with *Neisseria* at a high $OD_{600\ nm}$ (3.0 – 5.0). Unsurprisingly, much more growth can be seen in the overlay, resulting in the bacterial spot being much less visible, meaning that any areas of clearing / overgrowth directly over and around the spot would be much more difficult to determine and visualise.

The method that was used for the remaining overlay experiments was as follows:

10 µl spots of each isolate were placed in the centre of HBA plates, with additional streak plates prepared for culture inoculation. Plates were cultured overnight at 37 °C. The following day, liquid cultures of each genus were inoculated to an initial optical density (OD_{600nm}) of 0.05, measured using an Eppendorf Biophotometer spectrophotometer (Eppendorf, Germany). 1 ml culture samples were measured by OD_{600nm} every hour until they measured OD_{600nm} 0.5 – 1.0, when 200 µl of culture was added to 10 ml of molten 0.3 % Mueller-Hinton agar (MHA), mixed briefly and poured gently over the bacterial spots. Chemical control experiments were also carried out, replacing the bacterial spots with circles of Whatman filter paper soaked with 10 µl hydrogen peroxide (Sigma), at concentrations of 100 mM and 200 mM. All plates were incubated overnight at 37 °C in 5 % CO_2 and observed for areas of clearing / increased bacterial growth the following morning.

Figures 4.3 and 4.4 overleaf show representative photographs of results from overlay experiments between the genera *Neisseria* and *Veillonella*. In overlays where *Neisseria* is the bacterial spot and *Veillonella* is present in the overlay (Figure 4.3) and in the reverse combination, where *Veillonella* is the bacterial spot overlaid with *Neisseria* (Figure 4.4), no effect on bacterial overlay growth, either positive or negative, can be obviously visualised. This suggests that the two bacteria can inhabit independently of one another within the oral cavity. This is unsurprising, as *Neisseria* is an aerobic bacterium that can be facultatively anaerobic when required, whereas, *Veillonella* is a much stricter anaerobe (Breed et al., 1957, Smith, 1977).

Figure 4.3.

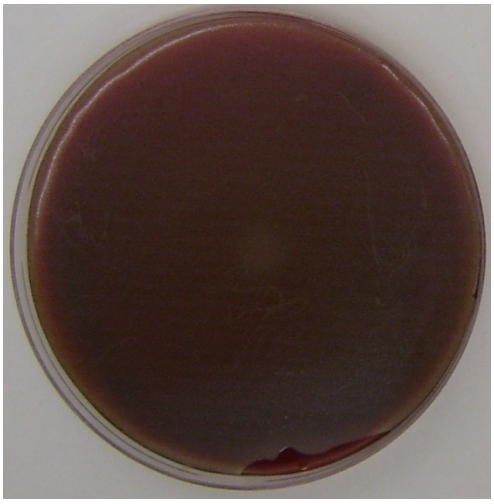


Figure 4.3. Photograph of a bacterial overlay where *Neisseria* is the bacterial spot plated on HBA and the overlay consists of 0.3 % MHA inoculated with *Veillonella*. Overlays were incubated at 37 °C in 5 % CO_2 and observed the following morning. No obvious areas or clearing or overgrowth of the *Veillonella* can be seen, indicating that there is no interaction strong enough to cause visible results between these two genera.

Figure 4.4.

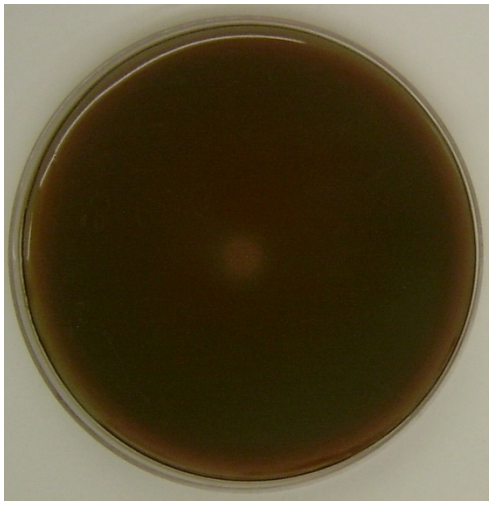


Figure 4.4. Photograph of a bacterial overlay where *Veillonella* is the bacterial spot plated on HBA and the overlay consists of 0.3 % MHA inoculated with *Neisseria*. Overlays were incubated at 37 °C in 5 % CO_2 and observed the following morning. No obvious areas or clearing or overgrowth of the *Neisseria* can be seen, indicating that there is no interaction strong enough to cause visible results between these two genera.

Figures 4.5 and 4.6 overleaf show representative photographs of results from bacterial overlay experiments with the genera *Veillonella* and s*treptococcus.* In overlay experiments where *Veillonella* is the bacterial spot and *Streptococcus* is present in the overlay (Figure 4.5), no obvious effect on bacterial growth can be visualised. This indicates that if the two genera are interacting, the effect of this interaction is not great enough to affect the growth of either genus.

Figure 4.6 shows the reverse overlay experiment, where *Streptococcus* is the bacterial spot and *Veillonella* is inoculated in the overlay. In contrast to the result shown in Figure 4.6, clearing of the overlay can be seen directly over and immediately around the bacterial spot, showing a lack of bacteria present in the overlay in this area. The bacterial spots measured approximately 5 mm in diameter and the areas of clearing were between 6 and 8 mm in diameter. These results indicate that the *Streptococcus* is killing the *Veillonella* directly above and in close proximity to it and furthermore, is continuing to prevent its re-growth over the time of incubation (12 – 16 hours).

Figure 4.5.

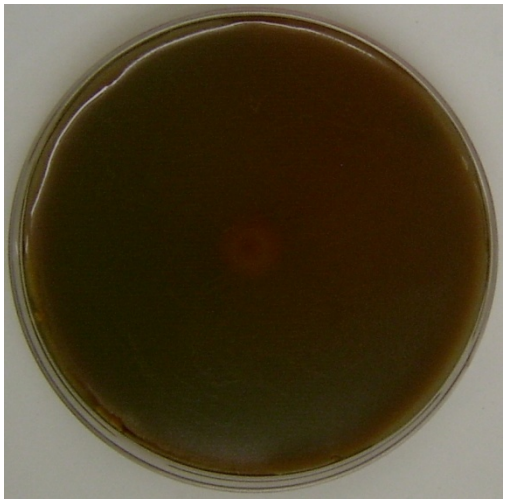


Figure 4.5. Photograph of a bacterial overlay where *Veillonella* is the bacterial spot plated on HBA and the overlay consists of 0.3 % MHA inoculated with *Streptococcus.* Overlays were incubated at 37 °C in 5 % CO_2 and observed the following morning. No obvious areas or clearing or overgrowth of the *Streptococcus* can be seen, indicating that there is no interaction strong enough to cause visible effects on growth between these two genera.

Figure 4.6.

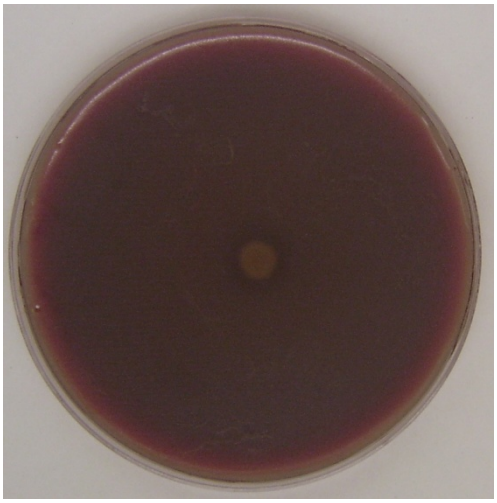


Figure 4.6. Photograph of a bacterial overlay where *Streptococcus* is the bacterial spot plated on HBA and the overlay consists of 0.3 % MHA inoculated with *Veillonella*. Overlays were incubated at 37 °C in 5 % CO_2 and observed the following morning. An area of clearing can be seen directly over and around the bacterial spot,typically measuring between 6 mm and 8 mm.

Figures 4.7 and 4.8 show representative photographs from bacterial overlay experiments involving the genera *Streptococcus* and *Neisseria*. Figure 4.7 shows the typical result of an overlay experiment where *Neisseria* is the bacterial spot and *Streptococcus* was inoculated into the overlay. No obvious clearing or overgrowth of the overlay can be seen over or around the spot, as was also the case when *Neisseria* was overlaid with *Veillonella* (Figure 4.3). This, once again, indicates that if there are interactions between *Neisseria* and *Streptococcus*, they are not strong enough to impact the growth of either genus in this experimental setup.

In contrast to this, Figure 4.8 shows the results from the reverse bacterial overlay, where *Streptococcus* is the bacterial spot and *Neisseria* is inoculated into the overlay. Once again, clearing can be seen directly over and immediately around the bacterial spot, as was also seen in previous overlays where *Streptococcus* was the bacterial spot (Figure 4.6). Bacterial spots measured approximately 5 mm in diameter and areas of clearing were typically 8 – 10 mm in diameter, larger than those seen in experiments where *Streptococcus* was overlaid with *Veillonella* (6 – 8 mm). This result indicates that there is an interaction between *Streptococcus* and *Neisseria*, resulting in *Neisseria* being killed by *Streptococcus* and its re-growth being prevented during the incubation period (12 – 16 hours).

Figure 4.7.

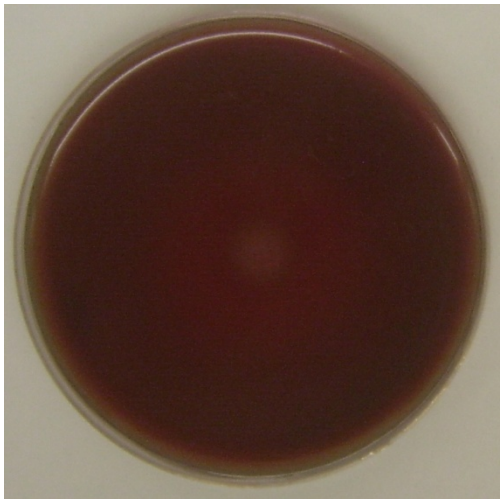


Figure 4.7. Photograph of a bacterial overlay where *Neisseria* is the bacterial spot plated on HBA and the overlay consists of 0.3 % MHA inoculated with *Streptococcus.* Overlays were incubated at 37 °C in 5 % CO_2 and observed the following morning. No obvious areas or clearing or overgrowth of the *Streptococcus* can be seen, indicating that there is no interaction strong enough to cause visible effects on growth between these two genera.

Figure 4.8.

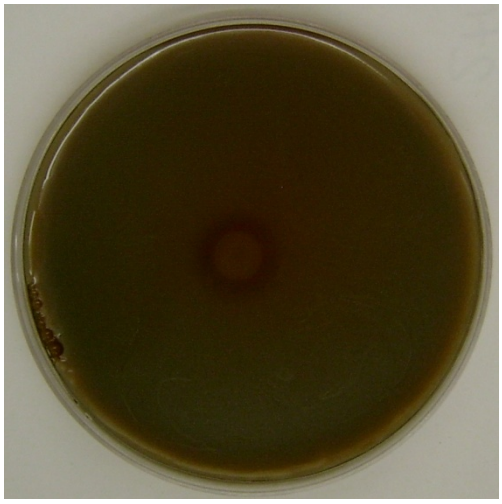


Figure 4.8. Photograph of a bacterial overlay where *Streptococcus* is the bacterial spot and the overlay consists of 0.3 % MHA inoculated with *Neisseria*. An area of clearing can be seen directly over and around the bacterial spot. Typically, areas of clearing measured between 6 mm and 10 mm.

In order to investigate the interactions further, the isolates were to be co-cultured in liquid media and their survival assessed by calculating the mean Colony Forming Units per millilitre (cfu/ml) for each isolate, over a

time course of growth. Before the co-culture experiments could be designed, it was necessary to establish the pattern of growth for each individual isolate over a time course. This would enable growth curves for each isolate to be obtained and allow any significant deviations in the normal growth pattern of each isolate to be visualised as a result of co-culture experiments.

To obtain the growth curves of the isolates in single culture, liquid cultures were inoculated in Mueller Hinton Broth (MHB) (Oxoid). *Neisseria* and *Streptococcus* isolates were cultured under aerobic conditions in 5 ml medium in 25 ml Universal tubes, at 37 °C with shaking at 200 rpm. Liquid cultures of *Veillonella* isolates were in 23 ml MHB in 25 ml Universal tubes, at 37 °C with shaking at 90 rpm, creating near anaerobic culture conditions. Liquid cultures were inoculated to an initial optical density (OD_{600nm}) of 0.05, measured using an Eppendorf Biophotometer spectrophotometer (Eppendorf, Germany). 1 ml culture samples were measured by OD_{600nm} every hour until the OD_{600nm} measured approximately 1.0, after which, 0.5 ml samples were taken every hour and diluted with a further 0.5 ml MHB. Cultures were performed in triplicate and growth curves were then plotted from the values obtained.

Figure 4.9 shows typical growth curves of the *Neisseria*, *Streptococcus* and *Veillonella* isolates. It was surprising to observe growth of *Veillonella*, as it was not cultured under strict anaerobic conditions. This indicates that *Veillonella* is not a strict anaerobe and is able to survive in the presence of oxygen. Possible reasons for this ability to survive in the presence of oxygen are discussed later in this chapter.

Figure 4.9.

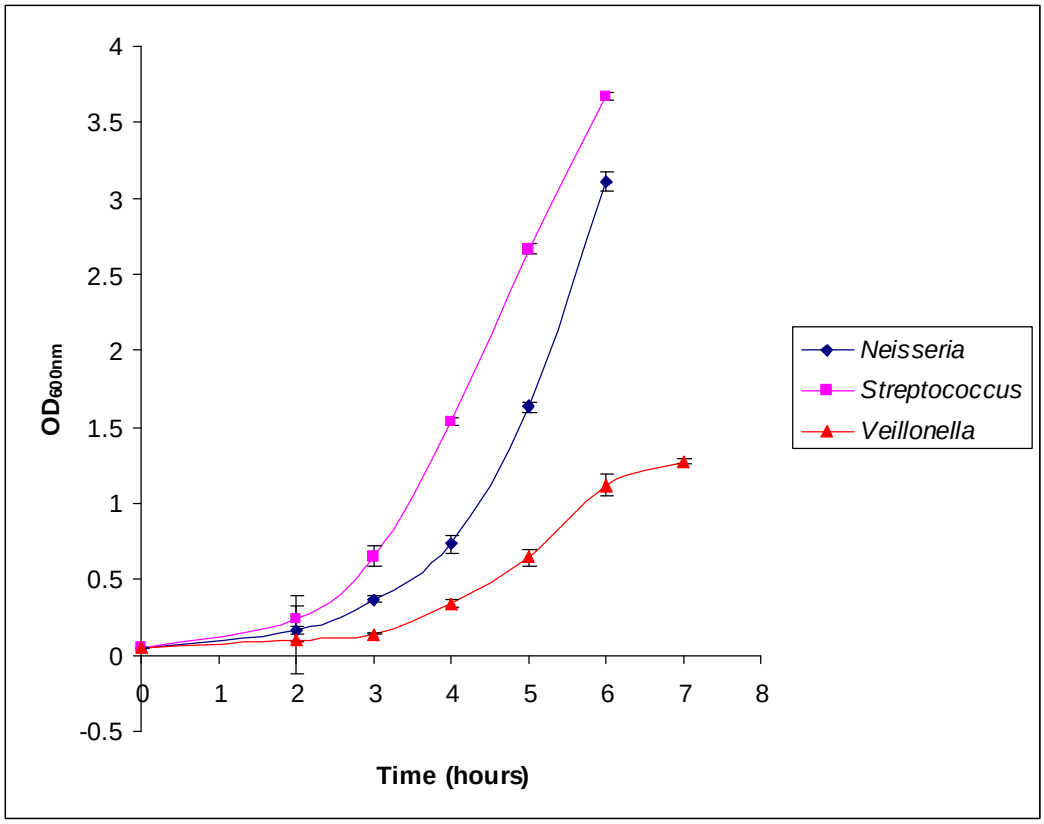


Figure 4.9. Graph showing the typical growth of each of the selected genera, over a 7 hour time course. The bacterial stocks used to inoculate each of the cultures were of the isolates obtained from mouth rinses during the diversity analyses. Error bars represent standard error. Both *Streptococcus* and *Neisseria* reached an OD_{600nm} of between 3.0 and 4.0 within 6 hours. Surprisingly, *Veillonella* also grew to an OD_{600nm} of 1.27 after 7 hours, showing that this isolate is not strictly anaerobic and can tolerate the presence of oxygen.

The strongest interaction observed during the bacterial overlay experiments was between *Neisseria* and *Streptococcus*, this indicates that the toxic effect of *Streptococcus* on *Neisseria* is the strongest. It was therefore decided that the interactions between *Neisseria* and *Streptococcus* should be investigated further. Liquid co-culture experiments were set up to investigate the interaction in terms of bacterial survival over several hours. Pre-cultures of *Neisseria* and *Streptococcus* were cultured as described above, until the start of log phase growth. Co-cultures were prepared in 5 ml MHB, with *Neisseria* cultures supplemented with 10 mM $NaHCO_3$. All cultures were at 37 °C with shaking at 200 rpm. Co-culture inocula were standardised by bacterial OD_{600nm}, with 20 µl of each bacterial pre-culture being inoculated into fresh MHB medium supplemented with 10 mM $NaHCO_3$. Each hour, 100 µl samples were removed from the co-culture and serially diluted in MHB to final dilutions of 10^{-4} and 10^{-5}. 100 µl samples of each dilution were plated in triplicate on HBA (prepared as in section 2.7) and incubated overnight at 37 °C in 5 % CO_2. Species were distinguished by colony morphology the following day, with each type of colony counted and Colony Forming Units per ml (CFU/ml) calculations determining bacterial survival.

Figure 4.10 overleaf shows the representative result of these liquid co-culture experiments. It can be seen that the *Neisseria* did not grow well in co-culture with the *Streptococcus* and after being in culture for 3 hours, the *Streptococcus* began to grow rapidly, whereas *Neisseria*l growth remains suppressed. This observation is consistent with that of the bacterial overlay experiments – *Streptococcus* is killing *Neisseria* and continuing to prevent its growth during the entire culture period.

Figure 4.10.

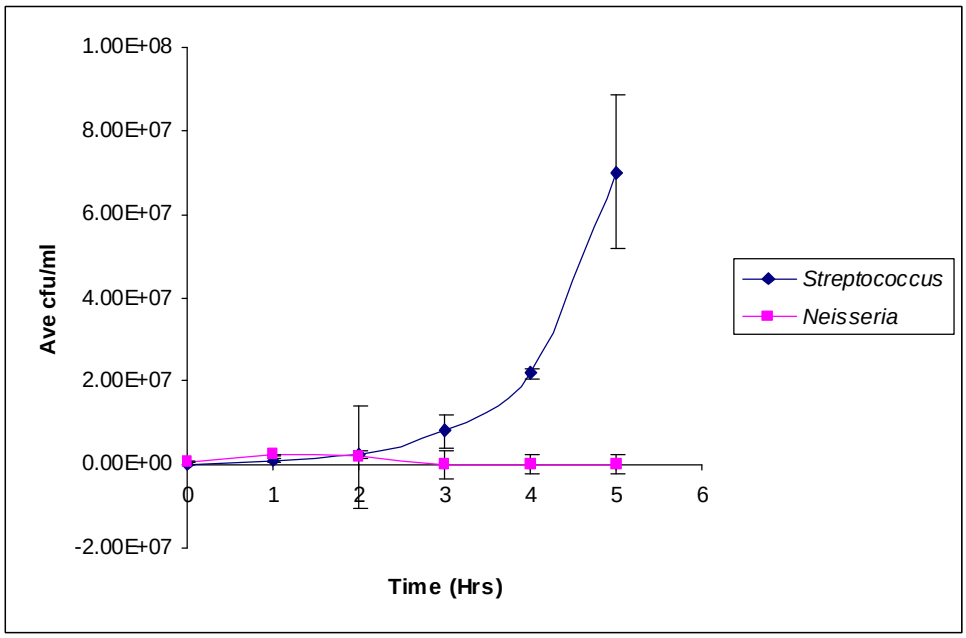


Figure 4.10. Graph showing mean survival as determined by CFU/ml calculations, of *Neisseria* and *Streptococcus* isolates during liquid co-culture experiments. Error bars represent standard error. The observations are consistent with those seen during bacterial overlay experiments, with *Neisseria* unable to grow in the presence of *Streptococcus*.

It is known that several strains of *Streptococcus* produce hydrogen peroxide as a metabolic by-product (Thomas and Pera, 1983, Vernazza and Melville, 1979). It was therefore hypothesised that the *Streptococcus* in the above experiments was producing hydrogen peroxide that was toxic to the *Neisseria*, resulting in the killing of *Neisseria* and continued suppression of its growth.

In order to test this hypothesis and also to be confident in the bacterial species used in these investigations, type strains from the genera *Neisseria* and *Streptococcus* were obtained. The MC58 strain of *Neisseria meningitidis* serogroup B was used, as the genome has been fully sequenced and annotated (Tettelin et al., 2000). *Streptococcus pneumoniae* ATCC 6303 was purchased from the American Type Culture Collection (Manassas, USA), which is a known hydrogen peroxide-producing strain, which allows the hypothesis above to be tested. This species has also been used in previous hydrogen peroxide studies (Pericone et al., 2000).

Liquid co-culture experiments were carried out by culturing these type strains as described above, with the addition of 10 mM $NaHCO_3$ in the *Neisseria* cultures and 210 U/ml catalase in the *Streptococcus* cultures. 20 µl of each single species culture was then inoculated into 5 ml fresh MHB medium, containing 10 mM $NaHCO_3$, ± 210 U/ml catalase. If the hypothesis above is correct, higher levels of growth of *Neisseria* would be seen in co-cultures containing catalase, as this would offer protection again the toxic hydrogen peroxide.

Figures 4.11 and 4.12 overleaf shows representative results of both liquid co-culture conditions. In the absence of catalase, MC58 grew well, but struggled to survive longer-term in the co-culture, being killed when *S. pneumoniae* growth increased at approximately 4 hours after co-culture inoculation. However, when the culture medium was supplemented with catalase, MC58 was able to grow rapidly and survived well when in culture with *S. pneumoniae*, considerably outgrowing the *Streptococcus*. The

protection offered to MC58 from the catalase agrees with the hypothesis above, supporting the idea that hydrogen peroxide is involved in the killing of *Neisseria* by *Streptococcus.*

Figure 4.11.

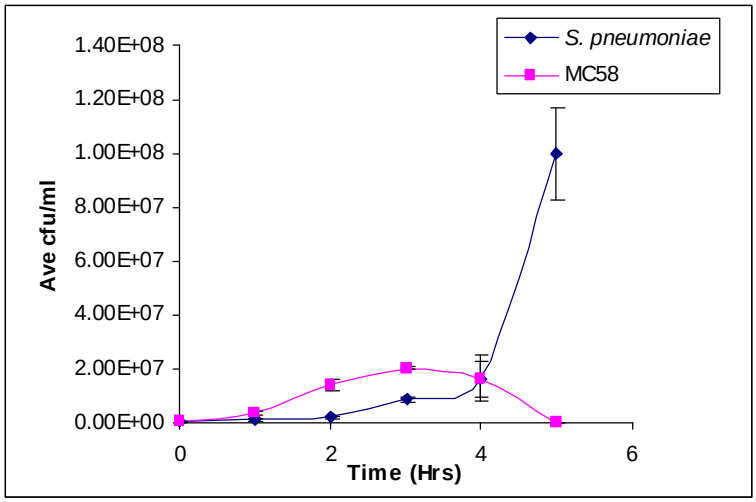


Figure 4.11. Results of liquid co-culture experiments between *N. meningitidis* MC58 and *S. pneumoniae*, in MHB containing 10 mM $NaHCO_3$, without the addition of catalase. MC58 grew in the co-culture, but was killed when *S. pneumoniae* grew exponentially after 4 hours in co-culture.

Figure 4.12.

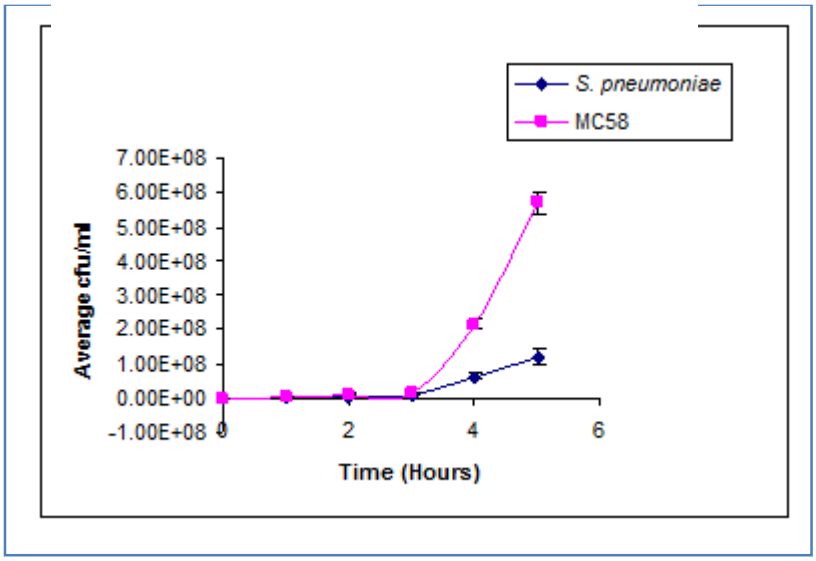


Figure 4.12. Results of liquid co-culture experiments between *N. meningitidis* MC58 and *S. pneumoniae*, in MHB, containing 10 mM $NaHCO_3$, with the addition of 210 U/ml catalase. In contrast to Figure 4.11, MC58 grew well in the co-culture and significantly outgrew *S. pneumoniae* from 3 hours after co-culture inoculation.

In order to further investigate the role of hydrogen peroxide in this interaction, it was necessary to determine the concentration of hydrogen peroxide produced by *S. pneumoniae*, both when in single species culture and in co-culture with *N. meningitidis.*

In order to measure the concentration of hydrogen peroxide produced by *S. pneumoniae* in single species culture, a standard curve was prepared from known concentrations of hydrogen peroxide (Sigma Aldrich) diluted in MHB and assayed using a Hydrogen Peroxide Assay Kit (National Diagnostics). Assay reagent was prepared from the kit according to the manufacturer's instructions. 0.9 ml of assay reagent was mixed thoroughly with 0.1 ml of known hydrogen peroxide concentration, before being maintained at room temperature for 30 minutes to allow complete colour development. All reactions were performed in triplicate before being measured spectrophotometrically by their absorbance at 560 nm and average readings plotted as a standard curve, which can be seen in Figure 4.13.

S. pneumoniae cultures were prepared as described previously, although cultures lacked the addition of catalase, and growth was measured by OD_{600nm} each hour over an 8 hour period. Alongside these growth readings, the concentration of hydrogen peroxide present in the culture medium was also measured by harvesting 0.5 ml culture samples by centrifugation (8000 rpm, 10 mins) and assaying the supernatant in triplicate as described above. Hydrogen peroxide assay results were interpolated from the standard curve shown in Figure 4.13, in order to determine the concentration of hydrogen peroxide in the culture medium.
hydrogen peroxide produced in liquid co-culture experiments containing *S. pneumoniae* and *N. meningitidis*, experiments were prepared as described previously, containing 10 mM $NaHCO_3$ only. 0.5 ml culture samples were harvested by centrifugation (8000 rpm, 10 mins) 1 hour post co-culture inoculation and then again 3 hours post inoculation. Assaying this co-culture would also provide an insight into how much hydrogen peroxide *N. meningitidis* can break down during the 3 hours of co-culture where the

bacteria can survive. Alongside the co-cultures, fresh, single species cultures of *S. pneumoniae* were also inoculated with and without 210 U/ml catalase, in order to assay and compare the hydrogen peroxide produced by the bacteria in single species and co-culture, when all cultures were prepared with the same inocula. All samples were assayed as described above. All assays were performed in triplicate and the calculated hydrogen peroxide concentrations, based on the standard curve were plotted.

Figure 4.13.

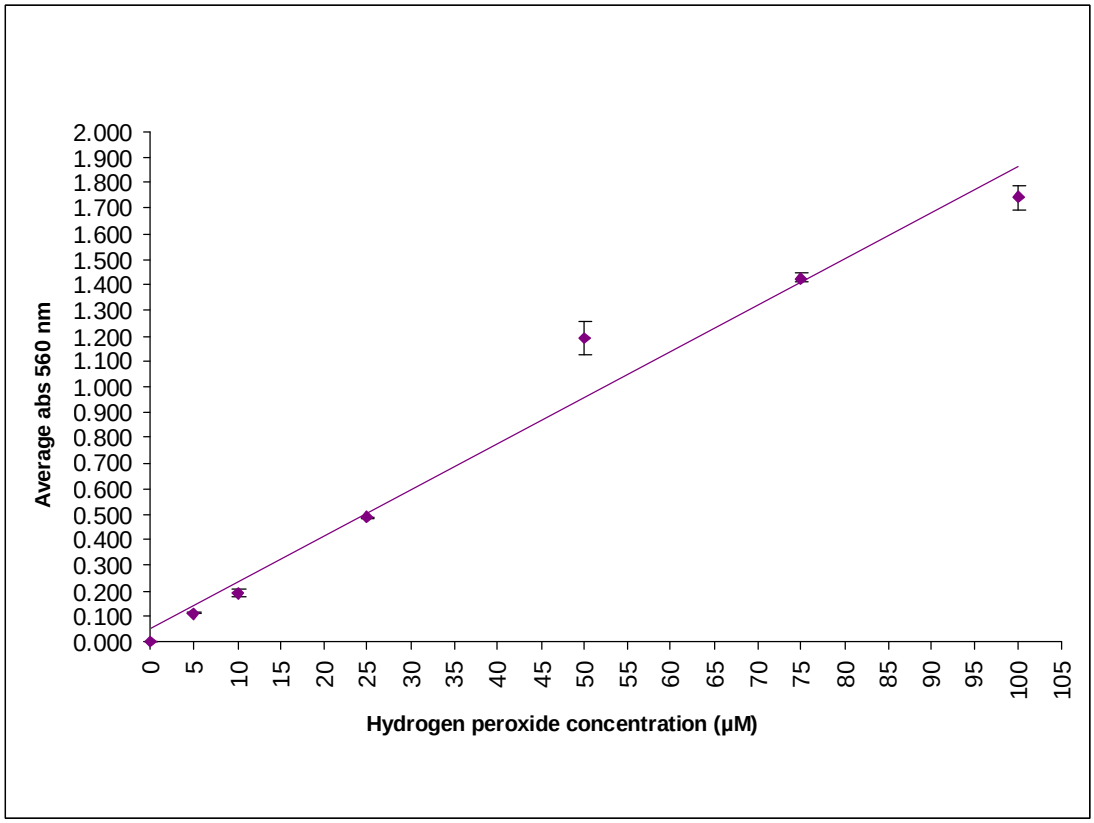


Figure 4.13. Standard curve produced from assaying known concentrations of hydrogen peroxide, with their corresponding absorbance at 560 nm. Samples were prepared using the Hydrogen Peroxide Assay Kit (National Diagnostics). Values plotted were average absorbance 560 nm, as calculated from assays performed in triplicate.

Figure 4.14 shows the concentration of hydrogen peroxide produced in a representative single species *S. pneumoniae* culture, lacking the presence of catalase, over an 8 hour growth period. Growth readings, as measured by OD_{600nm} are plotted against hydrogen peroxide concentrations. All readings were performed in triplicate and average values plotted. It can be seen that as the growth of *S. pneumoniae* increases, the hydrogen peroxide concentration also increases. This is unsurprising, as a higher number of hydrogen peroxide -producing bacteria should result in increased hydrogen peroxide concentration in the culture medium. As the bacteria enter stationary and death phase at approximately 6 hours, the hydrogen peroxide concentration also begins to decrease, suggesting that the concentration of hydrogen peroxide has built up to a level that is dangerous to the cells and begins to fall as the cells begin to die and do not produce any further hydrogen peroxide.

Although it is usual to culture *S. pneumoniae* in the presence of catalase in order to protect the bacteria from toxic effects of hydrogen peroxide, doing so in these experiments would significantly underestimate the levels of hydrogen peroxide produced in the culture, as the catalase would break the hydrogen peroxide down, resulting in inaccurate concentrations being determined. It is essential to gain a realistic insight into the potential hydrogen peroxide concentration produced by *S. pneumoniae* in its native environment, hence no catalase was present in these cultures.

Figure 4.14.

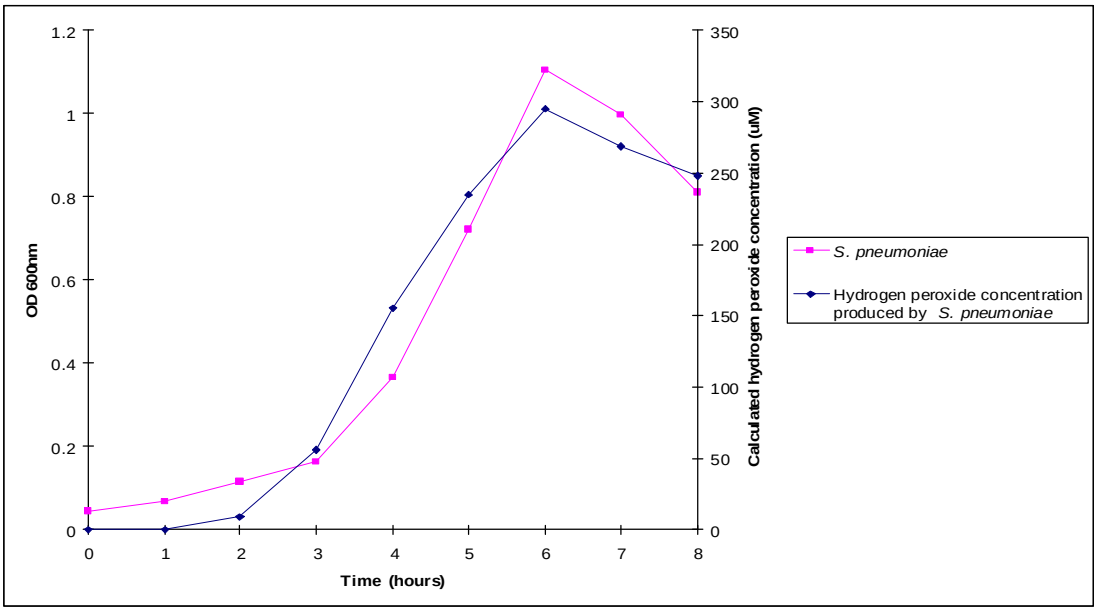


Figure 4.14. Comparison of growth and calculated hydrogen peroxide concentration in a *S. pneumoniae* single species culture, lacking catalase. Cultures were prepared and assayed as described above. Hydrogen peroxide concentration closely followed the pattern of growth, which is unsurprising.

Figure 4.15 shows the concentration of hydrogen peroxide produced in both a liquid co-culture experiment and freshly inoculated, single species cultures of *S. pneumoniae* lacking the presence of catalase. It can be hypothesised that liquid co-cultures containing *N. meningitidis* can break down a lot of the hydrogen peroxide that is being produced by *S. pneumoniae*. In order to investigate whether the differences in the hydrogen peroxide concentrations calculated from these experiments is statistically significant and therefore a true representation of what is occurring when the bacteria are under these culture conditions, t-tests were performed on each of the four data pairs. The results of these can be seen in Table 4.1 below.

Co-Culture Time Point (Hours)	**Calculated t-value**	**p-value**	**Are Hydrogen peroxide Concentrations Statistically Significant?**
3	3.21	0.033	Yes
4	7.83	0.001	Yes
5	17.63	0.000	Yes
6	30.86	0.000	Yes

Table 4.1. Results of t-tests performed on each of the four data pairs where hydrogen peroxide concentrations were calculated.

Figure 4.15.

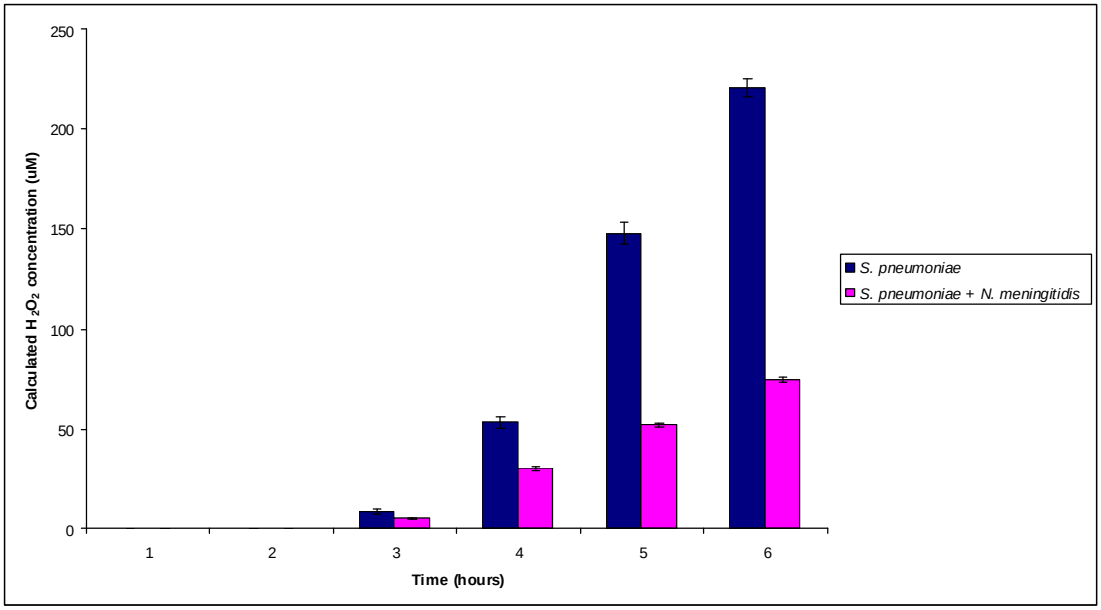


Figure 4.15. Hydrogen peroxide production as assayed from a liquid co-culture containing *S. pneumoniae* and *N. meningitidis* and from a single species culture containing *S. pneumoniae* only, inoculated at the same time as the co-culture, with an equal inoculum. Hydrogen peroxide levels rose in both cultures during the entirety of the experiment.

As shown in Table 4.1 and Figure 4.15, all differences between hydrogen peroxide concentrations calculated from single species and co-culture experiments were statistically significant. It is therefore reasonable to conclude that in liquid co-cultures, *Neisseria* was able to break down a significant amount of hydrogen peroxide produced by *Streptococcus.* Although it might seem reasonable to suggest that the breakdown of hydrogen peroxide by *N. meningitidis* aids its survival during co-culturing with *S. pneumoniae*, the fact remains that after approximately 4 hours of co-culturing with *S. pneumoniae*, *N. meningitidis* lost all viability. This suggests that the ability of *N. meningitidis* to metabolise peroxide is insufficient to ensure its survival under these culture conditions. Peroxide accumulation in the co-culture continued to be slower in the co-culture than the pure *S. pneumoniae* culture after 5 hours, indicating that peroxide removal may have continued after *N. meningitidis* wass no longer viable. The enzyme catalase is a dismutase that requires no other cellular components for activity, and so may continue to remove peroxide even after the cells that produced it have died.

4.3 Discussion.

4.3.1 Interactions between microbes in the oral cavity.

The oral cavity contains complex, multi-species microbial communities, with the total number of species colonising the mouth being greater than 700 (Aas et al., 2005, Paster et al., 2006). Therefore, it is reasonable to presume there are many interactions occurring between members of this complex community. The purpose of the experiments in this chapter was to investigate potential interactions, in terms of bacterial survival, between the three dominant genera identified from the diversity study carried out in Chapter 1. The strongest interaction observed during bacterial overlay experiments was between *Streptococcus* and *Neisseria* (see Figure 4.8) and we suggested that this interaction was possibly mediated by the metabolic by-production of hydrogen peroxide. Indeed, metabolic by-products are one of several mediators of bacterial interactions in the mouth, but there are also other mechanisms of interactions which are discussed here.

One important factor in determining the bacterial composition of a biofilm and microbial community is the availability of nutrients, coupled with the ability of the bacteria to metabolise these nutrients effectively (Kuramitsu et al., 2007). Nutrients are available from the intake of food during eating meals, saliva and production by other organisms as well as being present in dental plaque (Kuramitsu et al., 2007, Bowden, 1997). The presence of high levels of sucrose in many human diets is thought to give *Streptococcus mutans* an advantage over other oral bacteria, as it has the ability to metabolise sucrose very efficiently (Kuramitsu et al., 2007, Hamada and Slade, 1980). Furthermore, the metabolism of sucrose to lactic acid by *S. mutans* may give this species a competitive advantage over many bacteria, as it is able to survive well in the presence of this acid

(Dorana et al., 2004, Greiner, 1996) and may be relevant to its involvement in the onset of dental caries (Kuramitsu et al., 2007).

Other disease causing bacteria have formed symbiotic relationships in order to interact and co-inhabit niches within the mouth. *Porphyromonas gingivalis*, one of the major causes of periodontal disease, often coexists with other pathogenic bacteria, such as *Prevotella intermedia*, *Fusobacterium nucleatum*, *Tannerella forsythia*, and *Treponema denticola* (Moore et al., 1982, Dzink et al., 1988). It has been shown that *P. gingivalis* can use the succinate produced by *T. dentocola* in order to promote its own growth and in turn, that the isobutyric acid produced by *P. gingivalis* can aid the survival of *T. denticola* (Greiner, 1992). It has also been shown that extracts from *F. nucleatum* and *T. forsythia* can promote the growth of *P. gingivalis*, although the factors determining this are not yet known (Yoneda et al., 2001). These kinds of interactions may also enhance virulence of disease and evidence for this has been shown in murine oral abscess models (Yoneda et al., 2001, Kuramitsu et al., 2007). Although metabolic interactions may not fully explain this enhanced virulence, the interaction could play an important role in increasing the growth rate of pathogenic bacteria, thus accelerating the infection and disease process (Kuramitsu et al., 2007).

The proteins present in both saliva and gingival crevicular fluid are also sources of nutrients for bacteria in the mouth (Kuramitsu et al., 2007). Highly proteolytic species will therefore have a much greater advantage for growth where these kinds of nutrients are limiting (Kuramitsu et al., 2007). For this reason, it is thought that *P. gingivalis* can colonise the subgingival crevice and is involved in the onset of periodontitis (Mayrand et al., 1991, Potempa et al., 1995).

Mutualistic interactions also occur between organisms in the mouth. An example of this is between two organisms, *Actinomyces naeslundii* and *Streptococcus oralis*, who both appear to be dependent on saliva (Kuramitsu et al., 2007) and have both been identified in the diversity

study discussed in chapter 3. These two species coexist in dental plaque biofilms, but either species alone is a poor coloniser of saliva-coated tooth enamel (Palmer et al., 2001, Kuramitsu et al., 2007). Together, however, *A. naeslundii* and *S. oralis* can form plaque biofilms due to the combined metabolic activities of both species metabolising salivary nutrients (Kuramitsu et al., 2007).

Bacteria are also able to generate products which exert specific effects on other bacteria, with a good example of this being the expression of bacteriocins by some species (Kuramitsu et al., 2007). These are toxins that often have a narrow killing spectrum and inhibit the growth of related organisms (Cleveland et al., 2001, Chatterjee et al., 2005, Kuramitsu et al., 2007). Of all the oral bacteria, *Streptococci* have shown the greatest capacity for bacteriocin production (Nes et al., 2007), with *S. mutans* being shown to produce a number of these, also known as mutacins (Chikindas et al., 1995, Qi et al., 2001). The production of bacteriocins / mutacins may offer *Streptococci* a competitive survival advantage over other members of the oral microbial community.

Many oral bacteria have been shown to use bacteriocin-like compounds to compete with other species (Kuramitsu et al., 2007). Using overlay and agar diffusion methods, Teanpaisan et al. demonstrated the production of and sensitivity to bacteriocin-like activity among 44 strains of anaerobes, including *Porphyromonas* and *Prevotella*, which were isolated from periodontal sites (Teanpaisan et al., 1998, reviewed by Kuramitsu et al., 2007).

The production of and sensitivity to certain bacteriocin or bacteriocin-like activities among oral bacteria could enable bacteria to select their neighbours and interact with them, in order to increase disease virulence and progression, promote the establishment of biofilms and play an important role in the balance of the oral ecosystem (Kuramitsu et al., 2007). Indeed, it is possible that a bacteriocin, or bacteriocin-like activity was responsible for the interaction observed between the *Streptococcus*

and *Neisseria* isolates observed during bacterial overlay experiments in this chapter and it would be an interesting line of investigation to follow up during future studies.

It is also likely that the metabolic products of one organism have effects on other organisms within close proximity (Kuramitsu et al., 2007). It was this line of research that we chose to take during this study, with further investigations focusing on the effect of hydrogen peroxide, produced as a secondary metabolite by *Streptococcus*, exerting a toxic effect on *Neisseria*. Another example of hydrogen peroxide toxicity in oral microbial interactions is that taking place in dental plaque biofilms, with such biofilms containing relatively high proportions of *S. mutans* and low levels of *S. sanguinis* strains (Loesche et al., 1975). This is thought to be due to the ability of *S. mutans* to metabolise sugars to lactic acid. Since *S. mutans* strains are generally more aciduric than members of the *S. sanguinis* family, the production of lactic acid favours the growth of *S. mutans* (Kuramitsu et al., 2007). On the other hand, *S. sanguinis* is a producer of hydrogen peroxide, which has a toxic effect on *S. mutans*, as it does not express effective systems for breaking it down (Carlsson and Edlund, 1987, Kreth et al., 2005, Kuramitsu et al., 2007). Furthermore, many bacteria, including those which are associated with oral disease, are also sensitive to the toxic effect of hydrogen peroxide (Ihalin et al., 2001). Therefore, relatively high proportions of *S. sanguinis* are generally found in dental plaque with the absence or at least very low levels of *S. mutans* or pathogens such as *P. gingivalis* which is an indication of good oral health (Kuramitsu et al., 2007).

A further example of hydrogen peroxide toxicity in bacterial interactions within the oral cavity is that between *S. oligofermentans* and *S. mutans*. *S. oligofermentans* can metabolise the lactic acid produced by *S. mutants*, but in dental plaque biofilms, the former species is negatively correlated with the latter (Tong et al., 2007). It has also been shown that *S. oligofermentans* can inhibit the growth of *S. mutans* using a unique mechanism, where lactic acid is converted into hydrogen peroxide by

lactate oxidase (Tong et al., 2007). Hydrogen peroxide is extremely toxic to *S. mutans*, hence these species cannot coexist together in a plaque biofilm.

It is interesting to note that the production of hydrogen peroxide in plaque biofilms can also be an advantage to some species. Ramsey and Whiteley showed that during coculture experiments with *Streptococci*, the oral pathogen *Aggregatibacter actinomycetemcomitans* displayed enhanced resistance to killing by host innate immunity (Ramsey and Whiteley, 2009). They also deduced that the mechanism of resistance involved the sensing of the streptococcal metabolite hydrogen peroxide by *A. actinomycetemcomitans*, stimulating enhanced expression of the complement resistance protein ApiA, with the expression induced by OxyR (Ramsey and Whiteley, 2009). This study provided evidence for a link between oral bacterial communities and the Human innate immune response and provided an initial basis for understanding how this occurs.

As well as exerting toxic effects, metabolic products of one organism may promote the growth of other organisms during cooperative metabolic interactions (Kuramitsu et al., 2007). For example, the lactic acid produced by *S. mutans* can be readily metabolized by members of the *Veillonella* family (Mikx and van der Hoeven, 1975). A further example of this cooperative interaction is that of *F. nucleatum* and *P. intermedia* being able to produce ammonia and organic acids from subgingival crevicular fluid, thus lowering the pH of the environment and allowing the survival of *P. gingivalis* (Takahashi, 2003). This action also protects *P. gingivalis* from toxicity from acid-producing bacteria (Takahashi 2003, reviewed by Kuramitsu et al., 2007).

4.3.2 Bacterial overlay experiments reveal an interesting nsight into the ability of *Veillonella* to tolerate oxygen.

As shown in Figure 4.9, when cultured in the laboratory, *Veillonella* is able to grow in the presence of some oxygen. This is interesting to note, as it is thought that *Veillonella* is a strict anaerobe (Smith, 1977), even though it appears not to act as such in these experiments. Since the work discussed in this thesis was completed, the genome sequence of *Veillonella parvula* (strain DSM 2008) has been completed, which reveals the presence of genes encoding a cytochrome bd type oxidase in this 'anaerobe' (Gronow et al., 2010). This oxidase is encoded by genes Vpar_0477 and 0478 (Gronow et al., 2010). This type of oxidase is often associated with having a high oxygen affinity (Poole, 1995) and as such, may be used to scavenge oxygen to protect *V. parvula*, resulting in the bacteria being able to survive in the presence of a small amount of oxygen. It is perhaps not surprising to find such genes in the genome of an anaerobe, as no environment is free from the occasional presence of oxygen. However, the oxidative defences in anaerobes must be robust, as it is likely that such defences are critical for the movement of bacteria through aerobic environments to new anaerobic habitats (Imlay, 2008).

4.3.3 *Streptococcus pneumoniae* is a known hydrogen peroxide-producing strain used in previous studies and has been shown to kill other species.

Following observations made during bacterial overlay experiments between *Neisseria* and *Streptococcus* isolates, where *Streptcoccus* kills *Neisseria* and prevents its re-growth, it was decided to investigate whether hydrogen peroxide could be the mediator of this interaction. In order to investigate this further, type strains of both *Neisseria meningitidis* MC58 and *Streptococcus pneumoniae*. This *Streptococcus* species was chosen

as it is a known hydrogen peroxide producer (Annear and Dorman, 1952) and has been used previously in studies involving microbes that inhabit the Human nasopharynx (Pericone et al., 2000, Selva et al., 2009). The strain chosen, ATCC 6303, has previously been used by Bolm et al. (2004), where its ability to kill the nematode *Caenorhabditis elegans* was investigated as part of a larger study involving several Streptococcal species.

Bolm et al. (2004) performed both liquid and solid phase killing assays to test the ability of *S. pneumoniae* in killing *C. elegans*. During liquid phase killing assays, *C. elegans* larvae were added to log phase *S. pneumoniae* cultures and their viability scored (Bolm et al., 2004). Positive control experiments were carried out simultaneously, containing catalase to protect the larvae. During solid phase killing assays, late-phase *S. pneumoniae* cultures were plated on nematode growth medium agar, before incubation with *C. elegans* – vialbility of the nematodes was again scored (Bolm et al., 2004). The killing capacity of *S. pneumoniae*, alongside its hydrogen peroxide production, was assessed over a 24 hour time period, with hydrogen peroxide concentrations assayed by a horseradish peroxidase method (Bolm et al., 2004). Strain ATCC 6303 produced 1.7 mM concentration of hydrogen peroxide and was shown to kill 100 % of the *C. elegans* larvae (Bolm et al., 2004). The maximum hydrogen peroxide concentration was achieved after 6 hours (Bolm et al., 2004), which supports the length of the liquid co-cultures experiments performed in this chapter. These experiments were carried out over 5 hours post co-culture, which is sufficiently long to allow the hydrogen peroxide produced by *S. pneumoniae* to reach almost peak levels and exert the most toxicity over *N. meningitidis*.

Pericone et al. (2000) also performed liquid co-culture experiments to investigate the interaction between *S. pneumoniae* and other members of the Human nasopharynx, although the experiments mainly focused on the interaction with *Haemophilus influenzae*. Unlike liquid co-culture experiments carried out in this chapter, the experiments performed by

Pericone et al. were set up in 96-well plates and incubated prior to culturing on media to assess survival in terms of cfu/ml. Also, in contrast to experiments carried out in this chapter, the inocula of bacteria was controlled in terms of cfu/ml, instead of OD600nm. However, the bacterial inocula can be controlled in more than one way, so there is confidence in the methodology used in present experiments.

Pericone et al. (2000) also performed bacterial overlay experiments where *S. pneumoniae* was overlaid with *H. influenzae*. When such experiments were carried out, areas of clearing were seen, indicating that *S. pneumoniae* was having an inhibitory and bactericidal effect on *H. influenza*.When the reverse overlay experiments were carried out, no clearing was seen (Pericone et al., 2000). These observations are similar to those observed in this chapter, where *Streptococcus* isolates were overlaid with cultures inoculated with *Neisseria*, so once again there is confidence in the methodology that was developed and the observations that were made.

Furthermore, and in further support to the observations made in this chapter, Pericone et al. (2000) observed the killing of *N. meningitidis* when in co-culture with *S. pneumoniae*, with viability of *Neisseria* reduced by approximately 45 %.This result supports those presented in this chapter, although the interaction in the experiments in this chapter is seen to be stronger, with almost 100 % killing of *N. meningitis* by *S. pneumoniae*. However, the differences in the viability of *N. meningitidis* could be due to the differing co-culture methodologies used. Pericone et al. (2000) co-cultured *N. meningitidis* with *S. pneumoniae* culture supernatants, whereas, experiments in this chapter were performed from whole culture extracts, with no separation of cells from supernatant before samples were plated for viability. Moreover, the toxic compound responsible for the interactions observed by Pericone et al. (20000 was shown to be hydrogen peroxide.In spite of the differing methodologies, the same trend of results has been observed, indicating that there is confidence in the methodology used and there can be confidence in the hypothesis that

hydrogen peroxide is the mediator of the interaction between *Streptococcus* and *Neisseria*.

4.3.4 Concluding remarks.

The experiments performed in this chapter were designed and developed to investigate the interactions between the three dominant genera identified from the diversity study carried out in Chapter 3. Initial bacterial overlay experiments showed a strong interaction between *Streptococcus* and *Neisseria* isolates, with this interaction chosen to investigate further, by way of liquid co-culture experiments.

The liquid co-culture experiments, using type strains of each genus, gave further insight of this interaction and led to the hypothesis that the killing of *Neisseria* by *Streptococcus* was mediated by hydrogen peroxide, as *Neisseria* was protected and was able to survive with the addition of exogenous catalase.

The results observed in this chapter are in agreement with those previously published (Bolm et al., 2004, Pericone et al., 2000), suggesting that the production of hydrogen peroxide by *S. pneumoniae* may be used as a mechanism for competing with other oral and nasopharyngeal microbes (Pericone et al., 2000). The results also give strength and confidence to the results observed in this chapter and a good basis for investigating the interaction further, in terms of the key genes that may be involved and their regulation.

5. Determination and investigation of key genes involved in the interaction between *Streptococcus* and *Neisseria*.

5.1 Determination of key genes – Selection of *N. meningitidis* mutant strains.

It has been previously shown in Chapter 4 that *Streptococcus pneumoniae* has a negative effect on *Neisseria meningitidis* MC58 growth when the two bacteria are in liquid co-culture, but the addition of exogenous catalase exerts a protective effect on the *Neisseria*. Following these consistent observations, investigations into the possible key genes involved were carried out in order to gain more insight into the interaction between the two species at a genetic level.

As previously observed in Chapter 4, catalase provides protection to *Neisseria meningitidis* MC58 when in co-culture with *Streptococcus pneumoniae*. This supports the hypothesis that the interaction between these two species involves hydrogen peroxide, produced by the *Streptococcus*. It is therefore reasonable to presume that genes involved in peroxidase production / regulation may have roles in this interaction.

Several genes involved in peroxide stress resistance of *Neisseria meningitidis* MC58 were selected for examination in liquid co-culture experiments with *Streptococcus pneumoniae*, to investigate whether any of the mutants in these genes interacted in a different way to wild-type *Neisseria meningitidis* MC58.

The *katA::*Kan mutant was selected as this mutant contains a deletion in the *katA* gene which encodes the catalase enzyme. Therefore, no catalase is produced in this strain and the ability to break down hydrogen peroxide is greatly reduced. When in a co-culture experiment with *Streptococcus pneumoniae*, it is hypothesised that this mutant would

survive poorly and would be killed very quickly, more so than the wild-type *Neisseria*.

A second deletion mutant of *Neisseria meningitidis*, *oxyR::*Tet, was also selected to investigate in co-culture experiments with *Streptococcus pneumoniae*. This strain has a deletion of the OxyR regulator. It has been shown that in both *Neisseria gonorrhoeae* and wild-type *Neisseria meningitidis*, the OxyR protein represses *katA* gene expression and is only activated to derepress *katA* expression in the presence of a high level of peroxide (Seib et al., 2007, Ieva et al., 2008). Therefore, in an *oxyR* mutant, catalase is constitutively expressed, due to de-repression of the *katA* gene. This leads to the hypothesis that an *oxyR*::Tet mutant would be able to survive better than wild-type *Neisseria meningitidis* when in co-culture with *Streptococcus pneumoniae*, due to high levels of catalase being produced that can break down the toxic hydrogen peroxide produced by the *Streptococci*. This is indeed the case in *N. gonorrhoeae* where an *oxyR* mutant is highly resistant to hydrogen peroxide stress (Tseng et al., 2003). Contrastingly though, an *oxyR* gene deletion may prevent activation of the *prx* gene, which encodes the peroxidase peroxiredoxin, leading to a potential decrease in the ability to break down toxic hydrogen peroxide (Seib et al., 2007). Therefore, in co-culture with *S. pneumoniae*, the *oxyR::*Tet strain may be more, or less resistance to oxidative stress than the wild-type *Neisseria* strain.

A further deletion mutant, *prx::*Spec, was also selected for further investigation, since Prx is thought to be a peroxidase in Neisseria which is regulated by OxyR (Seib et al., 2007). The *prx::*Spec mutant carries a deletion of the peroxidase peroxiredoxin, reducing the ability of this mutant to break down toxic peroxides. However, it has been reported for *N. gonorrhoeae* that in a *prx* mutant, hydrogen peroxide resistance is elevated due to accumulated hydrogen peroxide allowing (presumably OxyR-dependent) de-repression of *katA* (Seib et al., 2007). Because of this, it can be hypothesised that *prx::*Spec may survive well in co-culture with *Streptococcus pneumoniae*. This strain of bacteria is resistant to

hydrogen peroxide, but tends to grow poorly. In spite of this, *prx::*Spec may survive at least as well as the wild-type *Neisseria meningitidis* in co-culture.

Two further peroxidase deletion mutants were also selected to investigate in co-culture experiments with *Streptococcus pneumoniae* - *bcp::*Ery and *gpxA::*Spec, encoding the peroxidases bacterioferritin co-migratory protein (*bcp*) and glutathione peroxidase (*gpxA*), respectively. A lack of these peroxidases in these two mutant strains would lead to decreased ability to break down toxic hydrogen peroxide, therefore, leading to the hypothesis that these two mutants will not survive as well as wild-type *Neisseria meningitidis* when in liquid co-culture experiments.

These mutant strains were constructed by previous members of the Moir lab – Dr Karin Heurlier, Dr Kristen Dyet and Mrs Diana Quinn. The constructions were deletion/insertions, where the gene of interest was amplified by PCR from *N. meningitidis* MC58, before being verified, cloned and transformed into *E. coli* DH5α competent cells. Antibiotic resistance cassettes were then excised from other vectors and inserted into the genes of interest via ligation at restriction enzyme sites. The antibiotic resistance cassette insertions disrupted the genes of interest, therefore causing them to be inactivated and creating the knock-out mutation of interest.

In order to be confident that the mutant strains were correct and suitable for use in further experiments, the mutations were verified by PCR. Primers were designed around the gene loci, which were determined by viewing the *N. meningitidis* genome sequence via the National Center for Biotechnology information (NCBI) website (http://www.ncbi.nlm.nih.gov/sites/genome). The nomenclature of each gene, along with its start and finish genome base pair (bp) location is shown in Table 5.1 overleaf. Primers were designed around these loci, with the expected size of the *N. meningitidis* MC58 gene fragment to be amplified by PCR also listed.

Table 5.1. Peroxidase stress response genes from *N. meningitidis* MC58.

Gene Name	**Nomenclature**	**Start (bp)**	**End (bp)**	**Expected** Wild-type **fragment size after PCR (bp)**
oxyR	NMB0173	167172	168092	910
bcp	NMB0750	780961	781401	423
katA	NMB0216	221438	222952	1441
prx (*grx*)	NMB0946	959840	960577	651
gpxA	NMB1621	1684618	1685151	483

As the genes of interest have been disrupted by insertion of an antibiotic resistance cassette, the PCR fragments amplified from the mutant strains will be much larger than the wild-type fragments.

5.2 Experimental results.

Figures 5.1 and 5.2 overleaf show PCR verification of the *bcp::*Ery and *gpxA::*Spec mutant strains, following amplification and separation of fragments by gel electrophoresis. Products were viewed on a 0.8 % agarose gel containing 0.21 ng/ml ethidium bromide, using a Syngene Gene Genius UV transilluminator gel documentation system.

In Figure 5.1, the wild-type *bcp* gene fragments can be seen at approximately 400 bp, with the *bcp::*Ery mutant gene fragment amplified at approximately 1.5 kb. This figure clearly shows the presence of the erythromycin antibiotic resistance cassette and verifies the construction.

In Figure 5.2, the wild-type *gpxA* gene fragment can be seen at approximately 500 bp, whereas, the *gpxA::*Spec mutant gene fragment was amplified at approximately 2.5 kb. Again, this clearly shows the presence of the antibiotic resistance cassette and verifies the mutant strain.

The absence of *katA*, *oxyR* and *prx* in their respective mutant strains was confirmed by RT-PCR. Primers for amplification of each of these genes are described in Table 2.11.1. Negligible amplification of each of these genes was found in RT-PCR experiments using cDNA generated from strains deficient in each of these genes.

Figure 5.1.

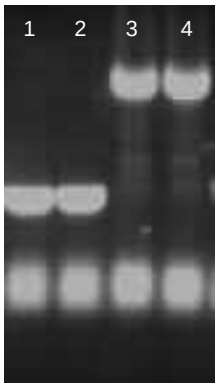


Figure 5.1. PCR verification of the *bcp::*Ery mutant strain. Lanes 1 and 2 show reactions with wild-type *N. meningitidis* MC58 template DNA. Lanes 3 and 4 represent two reactions with *bcp::*Ery template DNA. As predicted above, bands can be seen at around 400 bp (ladder not shown) in the wild-type bacteria, with much larger bands seen at around 1.5 kb in the mutant strain due to the insertion of the antibiotic resistance cassette during the construction of the mutant strain.

Figure 5.2.

Figure 5.2. PCR verification of the *gpxA::*Spec mutant strain. Lanes 1 and 2 show reactions with wild-type *N. meningitidis* MC58 template DNA. Lanes 3 and 4 represent two reactions with *gpxA::*Spec template DNA. As predicted above, bands can be seen at around 500 bp (ladder not shown) in the wild-type bacteria, with bands around 2.5 kb seen in the mutant strain due to the insertion of the 2 kb antibiotic resistance cassette during the construction of the mutation.

To assess the effect of *S. pneumoniae* on the growth of these various mutant strains of *N. meningitidis*, liquid co-culture experiments were set up as follows; pre-cultures of *S. pneumoniae* and *N. meningitdis* were cultured as described previously, until the start of log phase growth. Co-cultures were prepared in 5 ml MHB supplemented with 10 mM $NaHCO_3$ +/- 210 U/ml catalase and cultured at 37 °C with shaking at 200 rpm. Co-culture inocula were standardised by bacterial OD_{600nm}, with 20 µl of each bacterial pre-culture being inoculated. HBA plates containing the appropriate antibiotic to select for each type of mutant, as described in Table 2.8.1 were produced in advance of the experiment. Each hour, 100 µl co-culture samples were removed from the co-culture and serially diluted in MHB to final dilutions of 10^{-4} and 10^{-5}. 100 µl samples of each dilution were plated in triplicate on HBA plates + / - antibiotic and incubated overnight at 37 °C in 5 % CO_2. Species were distinguished by presence / absence on the relevant HBA plates the following day, with each colony counted and cfu/ml calculations determining bacterial survival. Negative control HBA plates were also prepared by streaking wild-type *Neisseria meningitidis* only onto each type of antibiotic plate and incubating as described above. These plates were verified following incubation and contained no bacterial growth (data not shown).

Figures 5.3 – 5.8 overleaf show results of liquid co-culture experiments with *Streptococcus pneumoniae* and the mutant *N. meningitidis* strains listed above. Surprisingly, all of the mutant strains, with the exception of *bcp::*Ery survived only as well as, or worse than the wild-type *Neisseria meningitidis* in the absence of catalase, with catalase exerting a strong protective effect on all the *Neisseria* strains. However, *bcp::*Ery, survived better than the wild-type strain in the absence of catalase in terms of cfu/ml, but was not killed by the *Streptococcus* and could maintain survival for the duration of the experiment, whereas the other mutant strains were killed after 3 – 4 hours. Once again, the presence of catalase exerted a protective effect on the *bcp::*Ery mutant strain, allowing it to outgrow the *Streptococcus* when in co-culture.

The results were all shown to be repeatable on several different occasions, confirming they are reliable and consistent observations that can be replicated.

Figure 5.3.

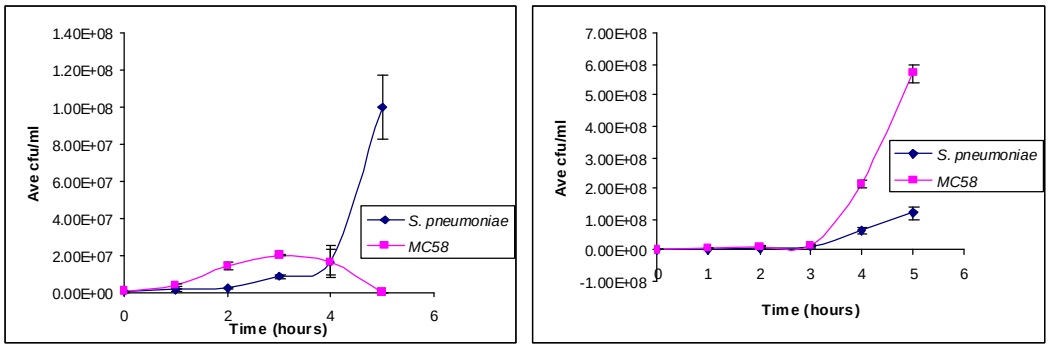


Figure 5.3. Results of liquid co-culture experiments between *N. meningitidis* MC58 and *S. pneumoniae*, with and without the addition of catalase. Error bars represent 1 standard error. When co-culture conditions lacked exogenous catalase, MC58 grew in the co-culture, but was killed after 4 hours in co-culture as *S. pneumoniae* growth continues. In contrast, in the presence of catalase, MC58 is able to grow well in the co-culture and significantly outgrows *S. pneumoniae* from 3 hours after co-culture inoculation.

Figure 5.4.

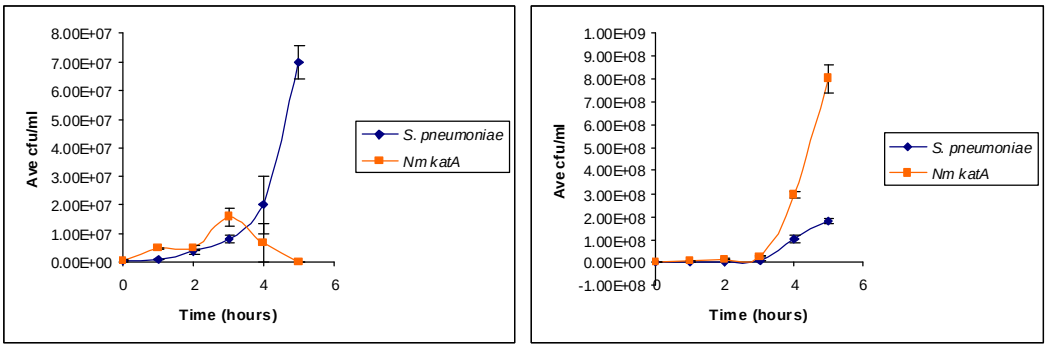


Figure 5.4. Results of liquid co-culture experiments between *katA::*Kan and *S. pneumoniae*, with and without the addition of catalase. Error bars represent 1 standard error. When co-culture conditions lacked exogenous catalase, *katA::*Kan grew in the co-culture, but was killed after 4 hours in co-culture as *S. pneumoniae* growth continues. In contrast, in the presence of catalase, *katA::*Kan grew well in the co-culture and significantly outgrew *S. pneumoniae* from 3 hours after co-culture inoculation.

Figure 5.5.

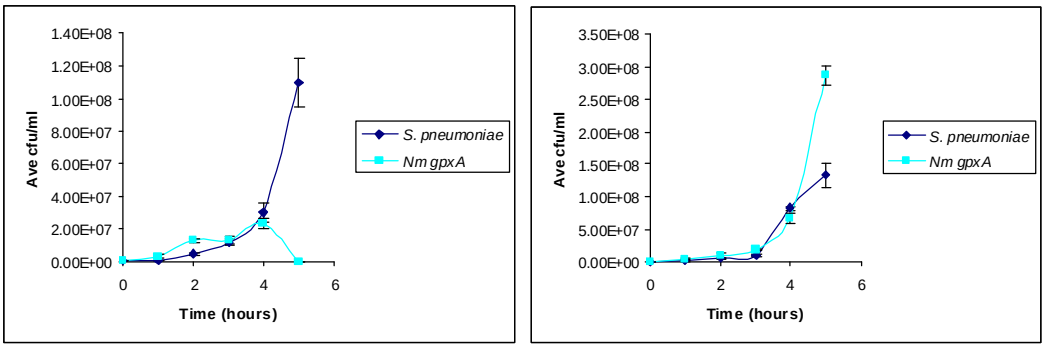


Figure 5.5. Results of liquid co-culture experiments between *gpxA::*Spec and *S. pneumoniae*, with and without the addition of catalase. Error bars represent 1 standard error. When co-culture conditions lacked exogenous calatalse, *gpxA::*Spec grew in the co-culture, but was killed after 4 hours in co-culture as *S. pneumoniae* growth continued. In contrast, in the presence of catalase, *gpxA::*Spec grew well in the co-culture and significantly outgrew *S. pneumoniae* from 3 hours after co-culture inoculation.

Figure 5.6.

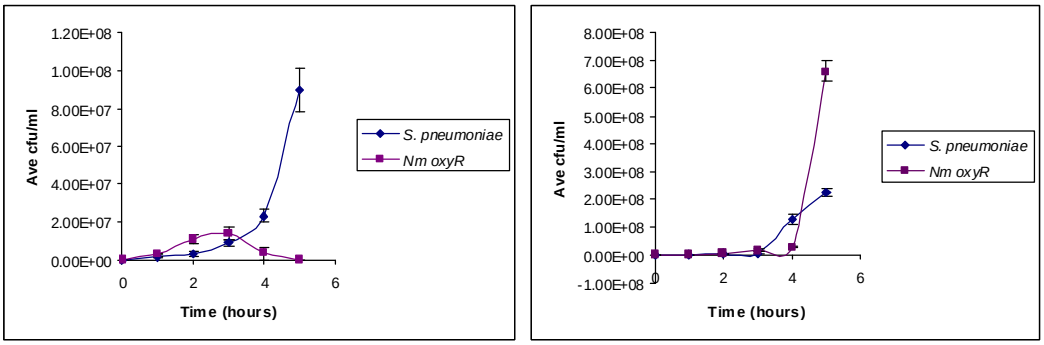


Figure 5.6. Results of liquid co-culture experiments between oxyR*::*Tet and *S. pneumoniae*, with and without the addition of catalase. Error bars represent 1 standard error. When co-culture conditions lacked exogenous catalase, oxyR*::*Tet grew in the co-culture, but was killed after 4 hours in co-culture as *S. pneumoniae* growth continued. oxyR*::*Tet may have survived less well than wild-type *Neisseria meningitidis* MC58 in these conditions. In contrast, in the presence of catalase, oxyR*::*Tet grew well in the co-culture and significantly outgrew *S. pneumoniae* from 3 hours after co-culture inoculation.

Figure 5.7.

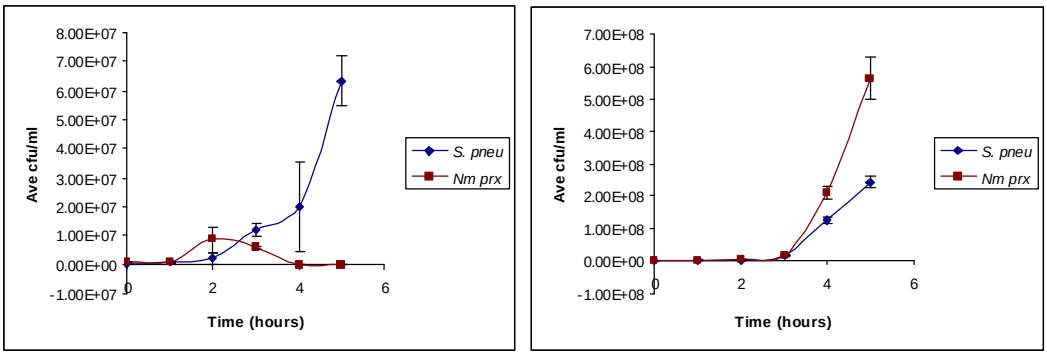


Figure 5.7. Results of liquid co-culture experiments between *prx::*Spec and *S. pneumoniae*, with and without the addition of catalase. Error bars represent 1 standard error. When co-culture conditions lacked exogenous calatalse, *prx::*Spec grew in the co-culture, but was killed after 3 - 4 hours in co-culture as *S. pneumoniae* growth continued. *prx::*Spec appeared to survive less well than wild-type *Neisseria meningitidis* MC58 in these conditions. In contrast, in the presence of catalase, *prx::*Spec grew well in the co-culture and significantly outgrew *S. pneumoniae* from 3 hours after co-culture inoculation.

Figure 5.8.

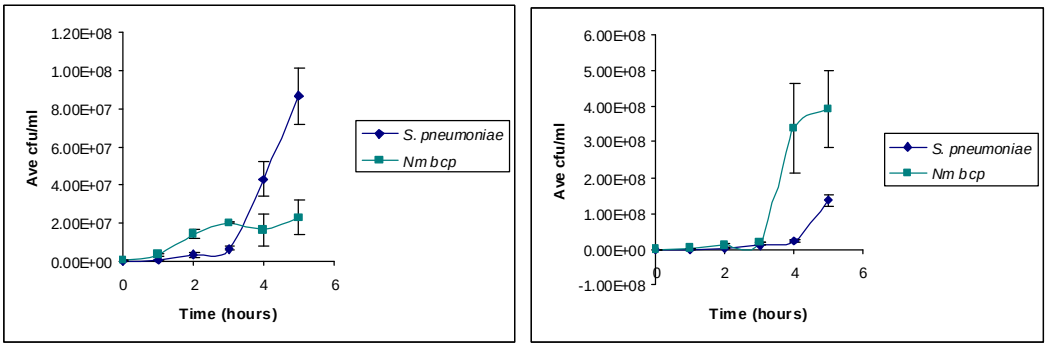


Figure 5.8. Results of liquid co-culture experiments between *bcp::*Ery and *S. pneumoniae*, with and without the addition of catalase. Error bars represent 1 standard error. In contrast to all other mutants investigated, *bcp::*Ery survived in the co-culture experiment, even as *S. pneumoniae* growth continued up to 1 x 10^{10} cfu/ml. The mutant *Neisseria* strain appeared to be continuing to survive and grew in the co-culture until the end of the experiment, 5 hours after co-culture inoculation. In the presence of catalase, *bcp::*Ery was able to grow well in the co-culture and significantly outgrew *S. pneumoniae* from 3 hours after co-culture inoculation.

The original hypotheses support *katA* being crucial for survival of *N. meningitidis* in co-culture with *S. pneumoniae*, but the data do not support this. Rather, Prx seems to be important since *prx* mutants survived particularly badly in the co-culture experiments.

The liquid co-culture experiments showed the *prx::*Spec mutant strain to be more sensitive to hydrogen peroxide than wild-type *N. meningitidis* MC58. This is very different to what was observed by Seib et al. (2007), when analysing *N. gonorrhoeae*. This previous study showed the expression of *prx* in *N. gonorrhoeae* to be increased 6-fold in the presence of 1mM hydrogen peroxide, with a *prx* mutant demonstrating increased resistant to hydrogen peroxide stress when compared to wild-type *N. gonorrhoeae* (Seib et al., 2007).

A further interesting result obtained from these experiments was obtained from co-cultures containing *S. pneumoniae* and the *bcp::*Ery mutant strain. These results suggest that a lack of the *bcp* gene, NMB0750, promoted survival of the mutant strain when in co-culture, as the *bcp::*Ery mutant strain survived much better in experiments when compared to the wild-type *Neisseria meningitidis* MC58. As a lack of the *bcp* gene promoted growth and survival of *Neisseria* in co-culture with *S. pneumoniae*, it suggests that another gene's expression is upregulated, to compensate for the lack of the Bcp protein being expressed. This upregulation may offer protection against the toxic peroxide, allowing the *bcp::*Ery mutant to survive and grow when in co-culture with *S. pneumoniae*.

A candidate gene that may be upregulated in a *bcp::*Ery mutant is *prx* (NMB0946), which could occur in an OxyR-dependent fashion. In order to investigate this further and to investigate whether liquid co-culture experiments between *S. pneumoniae* and mutant strains of *Neisseria meningitidis* MC58, containing deletions in the *bcp* gene and *prx* / *oxyR* gene, had any greater impact on the interaction already observed, double deletion mutant strains of *Neisseria meningitidis* MC58 were constructed by extraction and transformation of selected mutant chromosomal DNA,

according to a previously used method (Melanie Thomson, 2008, personal communication).

The resulting colonies were verified by PCR to confirm the double mutant constructions. Template DNA was prepared from a single *bcp::*Ery mutant and positive double mutant transformants.

Following amplification, products were viewed on a 0.8 % agarose gel containing 0.21 ng/ml ethidium bromide, using a Syngene Gene Genius UV transilluminator gel documentation system.

Figure 5.9 overleaf shows the results of the PCR verification, confirming that the *bcp::*Ery.*oxyR::*Tet double mutant construction was successful. The *bcp::*Ery.*prx::*Spec strain construction was verified by RT-PCR.

Figure 5.9.

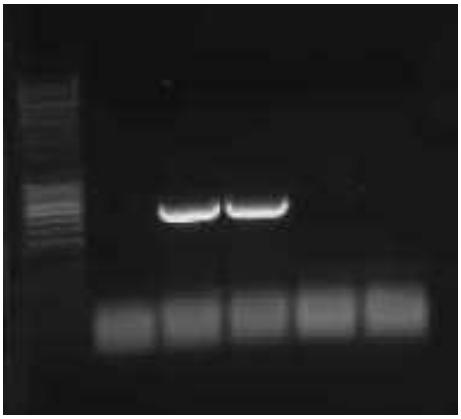


Figure 5.9. PCR verification of the *bcp::*Ery.*oxyR::*Tet double mutant construction. PCR products were visualised under UV light, following gel electrophoresis as described previously. The first lane next to the ladder was the negative control experiment, containing no PCR primers. The next two lanes showed the presence of the *oxyR* gene in a single *bcp::*Ery mutant, confirming that the primers amplified the desired gene. The final two lanes of the agarose gel show the result when the oxyR*::*Tet primers were used in a PCR reaction with the *bcp::*Ery.*oxyR::*Tet double mutant. This showed a lack of the *oxyR* fragments after amplification, which confirmed the *oxyR* knock-out due to the primer site deletion during the initial oxyR*::*Tet mutant construction. This confirmed the *oxyR* deletion is present in the double *bcp::*Ery.*oxyR::*Tet mutant.

As the *bcp::*Ery.*oxyR::*Tet double mutant strain lacks both a peroxidase and a regulator of the peroxidase stress response, and the *bcp::*Ery.*prx::*Spec double mutant strain lacks two types of peroxidase, it is hypothesised that the double mutant strains will not survive well when in liquid co-culture with *S. pneumoniae* and will survive less well than either the wild-type *Neisseria meningitidis* MC58 strain, or the respective single mutant strains of the bacteria.

In order to test this hypothesis and investigate the survival of the double mutants when in co-culture with *S. pneumoniae*, liquid co-cultures were set up as described previously, again with and without the presence of 210 U/ml Catalase. Control experiments between wild-type *Neisseria meningitidis* MC58 and *S. pneumoniae* were set up alongside the experiments between the double *Neisseria meningitidis* mutants and *S.*

pneumoniae. As per previous experiments (see Section 4.2), the co-culture inocula were controlled by OD_{600nm} and liquid co-cultures were sampled every hour for five hours post-inoculation, with average cfu/ml again used as a measure of bacterial survival.

Figures 5.10, 5.11 and 5.12 overleaf show results of these experiments. It can be seen that as hypothesised, the double mutant strains did not survive well when in liquid co-culture with *S. pneumoniae* and were unable to grow and survive in the absence of exogenous catalase. When catalase was present in the co-culture medium however, the double mutant strains were protected from the toxic hydrogen peroxide produced by the *Streptococcus* and were able to survive and grow. However, their growth was much slower than the wild-type *Neisseria* strain, with *bcp::*Ery.*oxyR::*Tet unable to outgrow the *Streptococcus* and *bcp::*Ery.*prx::*Spec growing at much the same rate as the *Streptococcus*. This indicates that the double knockout mutations may have some effect on the ability of the strains to grow when in competition with other strains or species.

Figure 5.10.

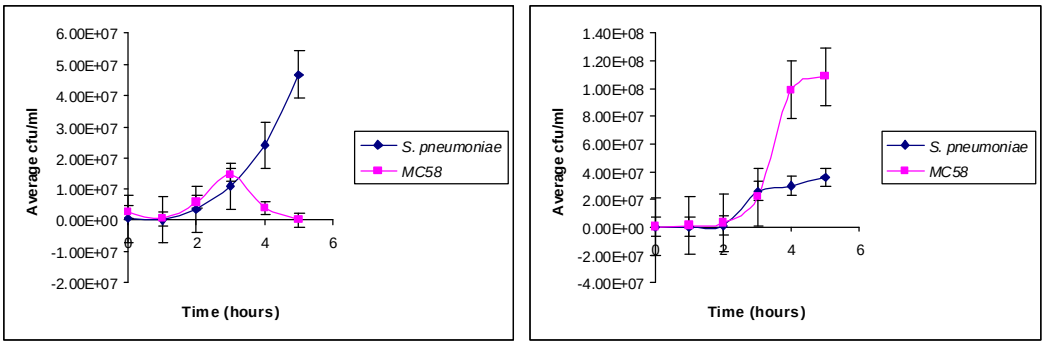


Figure 5.10. Results of liquid co-culture experiments between *N. meningitidis* MC58 and *S. pneumoniae*, with and without the addition of catalase. Error bars represent 1 standard error. When co-culture conditions lacked exogenous calatalse, MC58 grew in the co-culture, but was killed when *S. pneumoniae* grew exponentially between 3 and 4 hours in co-culture. In contrast, in the presence of catalase, MC58 grew well in the co-culture and significantly outgrew *S. pneumoniae* from 3 hours after co-culture inoculation.

Figure 5.11.

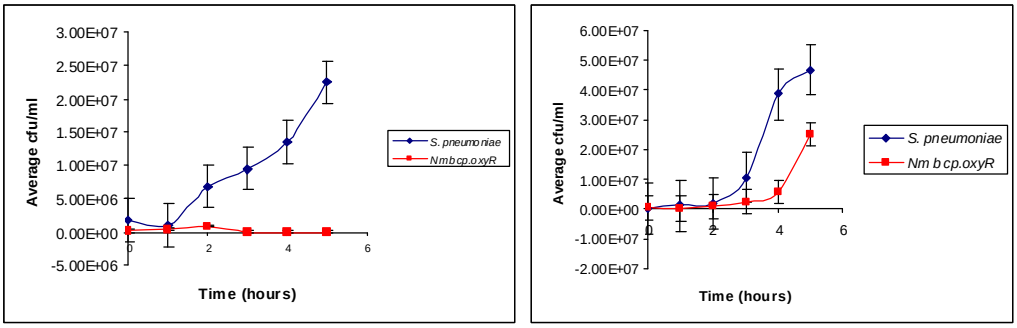


Figure 5.11. Results of liquid co-culture experiments between *Nm bcp::*Ery.*oxyR::*Tet and *S. pneumoniae*, with and without the addition of catalase. Error bars represent 1 standard error. When co-culture conditions lacked exogenous calatalse, the double mutant strain did not grow or survive well in the co-culture and was killed completely when *S. pneumoniae* grew exponentially from 2 hours in co-culture. In contrast, in the presence of catalase, *bcp::*Ery.*oxyR::*Tet was able to survive and grew from 4 hours in the co-culture. However, *bcp::*Ery.*oxyR::*Tet did not outgrow *S. pneumoniae*, indicating the double deletion mutation may have some negative effect on the ability of the strain to grow well.

Figure 5.12.

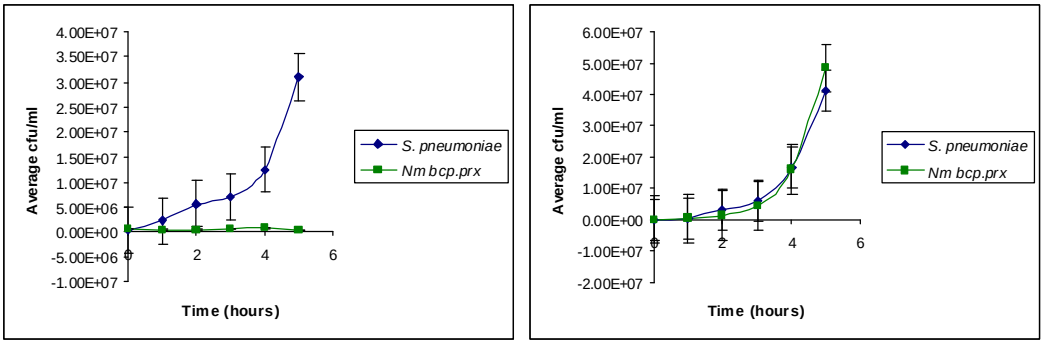


Figure 5.12. Results of liquid co-culture experiments between *bcp::*Ery.*prx::*Spec and *S. pneumoniae*, with and without the addition of catalase. Error bars represent 1 standard error. When co-culture conditions lacked exogenous calatalse, the double mutant strain did not grow or survive well in the co-culture. In contrast, in the presence of catalase, *bcp::*Ery.*prx::*Spec was able to survive and grew from 2 hours in the co-culture. This double mutant strain, in contrast to *bcp::*Ery.*oxyR::*Tet, was able to grow well in the presence of catalase and grew at approximately the same rate as the *Streptococcus*, having slightly outgrown it at the end of the experiment.

Having now obtained some insight into the ability of various *N. meningitidis* strains to survive when in liquid co-culture with *S. pneumoniae*, it was decided to determine whether treatment with aliquots of exogenous hydrogen peroxide would have the same effect.

As the *katA::*Kan mutant has a deletion in the *katA* gene which encodes the catalase enzyme, no catalase is produced in this strain and the ability to break down hydrogen peroxide was greatly reduced. This mutant survived poorly when in co-culture with *S. pneumoniae*, although no worse than the wild-type strain. Therefore, it is hypothesised that this strain will not survive when exogenous hydrogen peroxide is added to a single species culture.

*oxyR::*Tet contains a deletion of the OxyR regulator. In wild-type *Neisseria*, OxyR represses *katA* expression, therefore, as described previously, in the mutant catalase is constitutively expressed. However, as also previously described, the *prx* gene may not be activated in this strain, leading to a decrease in the ability to break down the hydrogen peroxide. Additionally, previous co-culture experiments showed that the *oxyR::*Tet strain did not survive any better than the wild-type strain, so this mutant may or may not show increased resistance in these experiments.

The *prx::*Spec mutant carries a deletion of the peroxidase peroxiredoxin, allowing peroxide levels accumulate to a sufficient level that *oxyR* becomes active and de-represses *katA*, increasing catalase expression. Because of this, it is hypothesised that *prx::*Spec may survive well in the presence of exogenous catalase.

*bcp::*Ery contains a deletion of the *bcp* gene, encoding the peroxidase bacterioferritin co-migratory protein. A lack of peroxidase in this mutant would lead to the hypothesis that this strain would survive poorly in the presence of hydrogen peroxide. However, from the previous liquid co-culture experiments, this strain was the only strain to survive when in co-culture with *S. pneumoniae*; therefore, it is hypothesised that the mutant

will also survive well when exogenous hydrogen peroxide is added to the single species culture.

The *gpxA::*Spec mutant has a deletion in glutathione peroxidase, therefore lacking an ability to break down toxic hydrogen peroxide. It is therefore hypothesised that this mutant will not survive well in the presence of exogenous hydrogen peroxide.

Both the newly constructed *bcp::*Ery.*oxyR*::Tet, lacking both a peroxidase and a peroxidase stress response regulator and *bcp::*Ery.*prx*::Spec, lacking two peroxidases, strains have already been shown to survive very poorly when in co-culture with *S. pneumoniae*. Therefore, it is hypothesised that both of the double mutant strains will not survive well when exogenous hydrogen peroxide is added to single species cultures of these strains.

To investigate the hypotheses above, single species cultures were prepared as described previously and the OD_{600nm} measured every hour until mid-log phase was reached. 1 mM hydrogen peroxide was then added to the cultures and 100 µl samples taken every 15 mins for 1 hour. Samples were serially diluted in MHB to a final dilution of 10^{-4}, before being plated in triplicate on HBA plates, with relevant antibiotics where necessary. All plates were incubated at 37 °C overnight, in 5 % CO_2. Resulting colonies were counted the following morning and cfu/ml calculations were performed to assess bacterial survival.

Figure 5.13 overleaf shows results of the above experiment. It can be seen that all *Neisseria* strains analysed confirmed the hypotheses above, apart from the two double mutant strains, *bcp::*Ery.*oxyR*::Tet and *bcp::*Ery.*prx::*Spec. Possible reasons for these results not agreeing with the hypotheses are discussed further in this chapter. The single mutant strain *bcp::*Ery did not survive as well as in the liquid co-culture experiments, but was not killed completely during the course of the sensitivity assay, showing that it may be more hydrogen peroxide resistant

than the *katA*::Kan, *gpxA*::Spec and wild-type MC58 strains. This supports the results obtained from the previous liquid co-culture experiments carried out.

Figure 5.13.

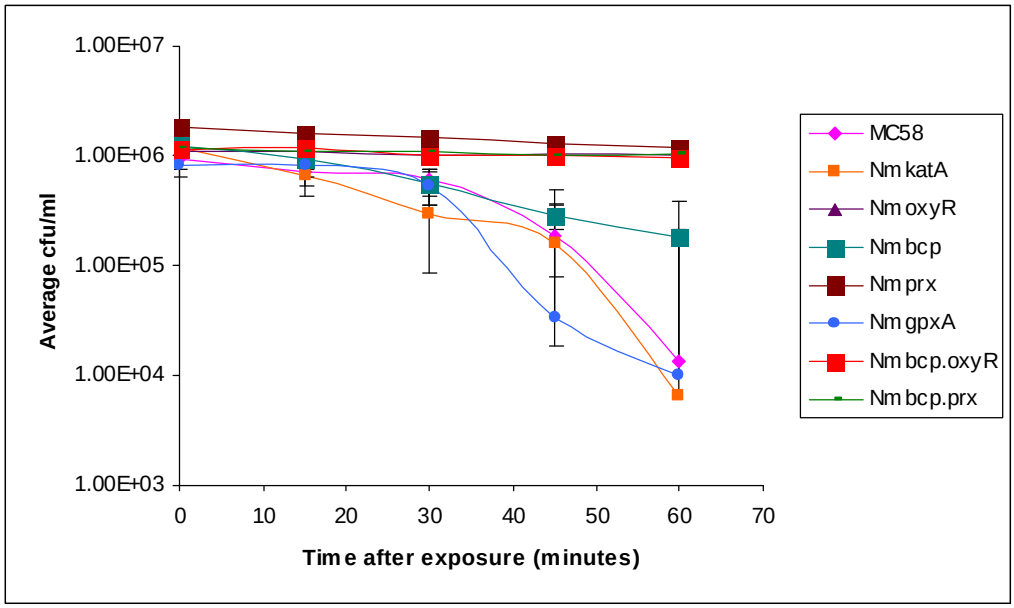


Figure 5.13. Results of hydrogen peroxide sensitivity assays performed on wild-type *N. meningitidis* single mutant strains and the newly constructed double mutant strains, shown on a logarithmic scale with standard error bars. All strains except *prx::*Spec did not survive during the experiment and all but *bcp::*Ery were killed by the addition of the hydrogen peroxide. *prx::*Spec did survive in the presence of the hydrogen peroxide. The *bcp::*Ery strain, although decreasing in average cfu/ml over the course of the assay, was not killed, showing an increased resistance to hydrogen peroxide compared to the other single species mutant strains. The two strains that deviated from the hypotheses were the newly constructed *bcp::*Ery.*oxyR* and *bcp::*Ery.*prx::*Spec strains, which both survived well in the presence of the hydrogen peroxide.

Following Real Time PCR (RT-PCR) experiments carried out previously, it was observed that when wild-type *N. gonorrhoeae* was exposed to low-level hydrogen peroxide, the expression of the *katA* gene was dramatically increased (Seib et al., 2007). This indicates that, at this exposure, the *katA* gene was being derepressed by activation of the *oxyR* gene product and catalase was being produced to provide protection against the toxic hydrogen peroxide.

In light of this observation, the following hypothesis was formed and tested with further hydrogen peroxide sensitivity assays;

Wild-type *N. meningitidis* MC58 is conditioned to low-level hydrogen peroxide by the up-regulation and de-repression of *katA* gene expression. Therefore, if pre-treated with low-level hydrogen peroxide, *N. meningitidis* MC58 should be able to survive the addition of high-level (1 mM) hydrogen peroxide. The k*atA::*Kan mutant strain will be unable to survive in the presence of high-level hydrogen peroxide, regardless of the concentration of the pre-treatment.

MC58 and *katA*::Kan strains were cultured as described previously to mid-log phase, where they were pre-treated with 20 µM or 50 µM hydrogen peroxide for 1 hour, before being exposed to 1 mM hydrogen peroxide for a further hour. Samples were taken every 15 minutes and treated as described previously throughout the 2 hour exposure period. Bacterial survival was measured by average cfu/ml calculations.

Figure 5.14 overleaf shows the results of this experiment. As hypothesised above, *N. meningitidis* MC58 survived much better in the presence of high-level hydrogen peroxide than in the previous sensitivity assay (see Figure 5.13) and survived much better than *katA::*Kan. It did not appear to matter which pre-treatment hydrogen peroxide concentration the bacteria are exposed to, as both concentrations used in this assay provided protection to *N. meningitidis* MC58 when the bacteria were exposed to high-level hydrogen peroxide.

Figure 5.14.

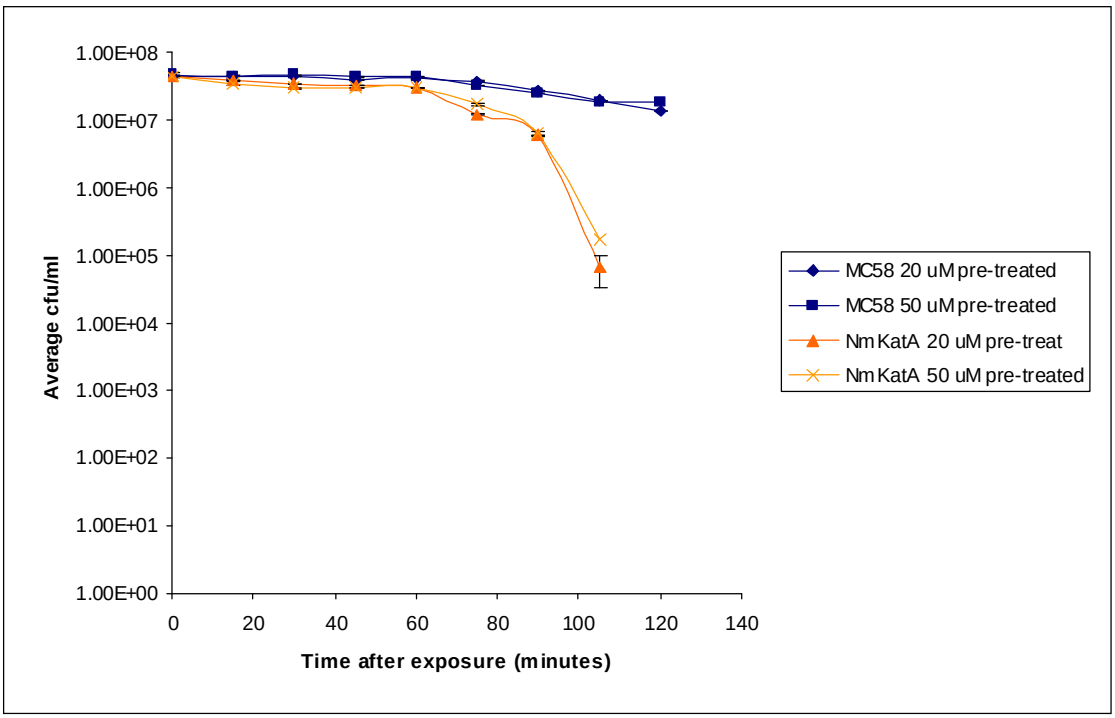


Figure 5.14. Results from further hydrogen peroxide sensitivity assays with pre-treatment to low-level hydrogen peroxide, shown on a log scale with standard error bars Following pre-treatment, MC58 survived much better than *katA::*Kan when exposed to high-level hydrogen peroxide. In contrast to the hypothesis above, the concentration of the pre-treatment did not greatly affect bacterial survival in the presence of high-level hydrogen peroxide.

As results shown so far in the chapter indicate, both single mutant *Neisseria* strains deficient in *bcp* and *prx*, respectively, resulted in behaviour that is very different to that of wild-type *N. meningitidis* MC58 when in liquid co-culture experiments with *S. pneumoniae*. In light of these observations, hydrogen peroxide concentrations were measured when these two mutant strains were in liquid co-culture experiments with *S. pnuemoniae*.

Liquid co-culture experiments were performed as described previously, with samples taken every hour over the co-culture period, for hydrogen peroxide concentration assaying. Assays were performed in triplicate and average calculated concentrations determined for each co-culture combination, at each time point.

Figure 5.15 overleaf shows the results of this experiment. During the course of culturing either alone, or in co-culture with the various *N. meningitidis* strains, hydrogen peroxide accumulated. In the presence of exogenous catalase, no peroxide could be measured. The presence of *N. meningitidis* decreased the measured amount of hydrogen peroxide at all time points, consistent with *N. meningitidis* actively removing peroxide. Co-cultures between *S. pneumoniae* and *N. meningitidis* strains deficient in either *prx* or *bcp* had lower levels of peroxide accumulation than with the wild-type *N. meningitidis*, indicating that the absence of these two genes was compensated for by elevated peroxide metabolism via alternative systems.

Figure 5.15.

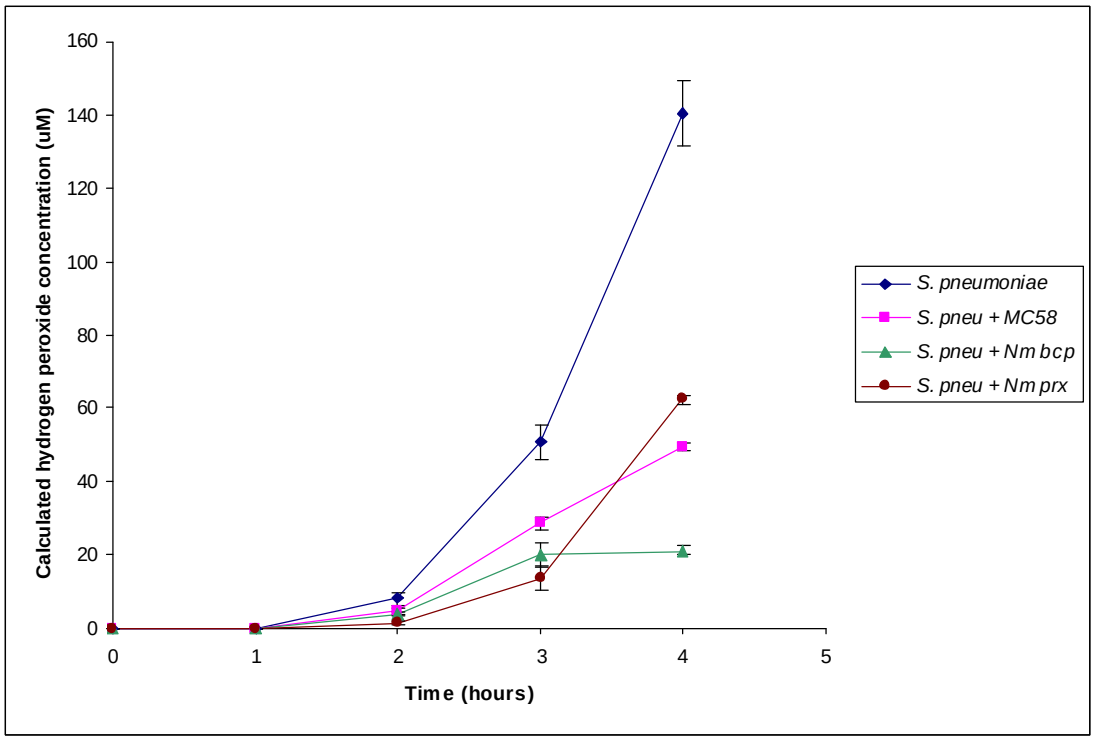


Figure 5.15. Hydrogen peroxide concentrations in liquid co-culture experiments. Error bars represent 1 standard error. The presence of various *N. meningitidis* strains decreased the measured amount of hydrogen peroxide at all time points. Co-cultures between *S. pneumoniae* and *N. meningitidis* strains deficient in either *prx* or *bcp* had lower levels of peroxide accumulation than with the wild-type *N. meningitidis*,.

To further investigate the involvement and regulation of the genes involved in the peroxidase stress response of *N. meningitidis*, the impact of genotype and environment on transcription of *prx*, *katA*, *bcp* and *gpxA* was assessed by RT-PCR.

N. meningitidis strains of interest were cultured as described previously, before RNA extraction and cDNA preparations were obtained for use in RT-PCR experiments. Detailed methodologies for each step can be found in the Materials and Methods (Section 2.10).

Following RT-PCR analysis, the number of cycles taken to amplify each gene to a threshold level of expression was calculated and the data manipulated as shown below, in order to determine the relative expression of the key genes of interest, compared to their expression in wild-type *N. meningitidis* MC58, normalised against expression of an endogenous constitutively expressed gene, *metK*. Relative increase in expression of gene A, ΔE_A, was calculated in terms of cycles to amplify a given gene in a given strain ($C_{gene,strain}$) as follows:

$$Log_2 (\Delta E_A) = (C_{A,MC58} - C_{A,mutant}) - (C_{metK,MC58} - C_{metK,mutant})$$

Figure 5.16 shows the results of these RT-PCR experiments. It can be seen that mutants deficient in *oxyR* displayed elevated expression of the gene *katA*, encoding catalase. This is in keeping with previous observations in *N. gonorrhoeae* (Tseng et al., 2003) and *N. meningitidis* (Ieva et al., 2008) that OxyR is a repressor of *katA* expression in the absence of peroxide. Furthermore, *prx* mutants also showed elevated expression of *katA*. This may be due to *prx* mutant strains being under increased levels of oxidative stress, thus leading to OxyR-dependent de-repression of *katA* under supposedly non-stressful growth conditions. This is in keeping with the observation made by Seib et al. (2007) that, in *N. gonorrhoeae*, catalase is upregulated in *prx* mutants. The up-regulation of catalase production in *oxyR* and *prx* mutant strains serves as a reasonable explanation for the resistance of these mutant strains to a

burst of hydrogen peroxide (see Figure 5.14), with catalase being required to deal with high concentrations of peroxide, and so the mutant strains deficient in *prx* or *oxyR* survive effectively in the hydrogen peroxide burst assay compared to wild-type and *katA* mutant strains.

Figure 5.16 also shows that *bcp*, *prx* and *gpxA* were all regulated in a peroxide / *oxyR* dependent manner. Expression of *bcp*, *prx* and *gpxA* were all significantly decreased in an *oxyR* mutant, indicating that this regulator is an activator of the peroxidase gene expression. Microarray studies with *N. gonorrhoea*e previously identified *prx* as part of the *oxyR* regulon (Seib et al., 2007), but not *bcp*; *gpxA* is absent from *N. gonorrhoeae*. Furthermore, mutating *prx* caused elevation of the expression of *bcp* and *gpxA*, giving further support to the notion that *N. meningitidis* cells deficient in *prx* are under oxidative stress. Mutating *bcp* may have increased expression of *prx* or *gpxA* but this was not statistically significant. In the presence of hydrogen peroxide, all peroxidase genes were upregulated, but *prx* expression was up-regulated further in a *bcp*::Ery mutant than in the wild type MC58 strain, as shown in RT-PCR experiments carried out on these strains after exposure to 50 µM hydrogen peroxide.

Figure 5.16.

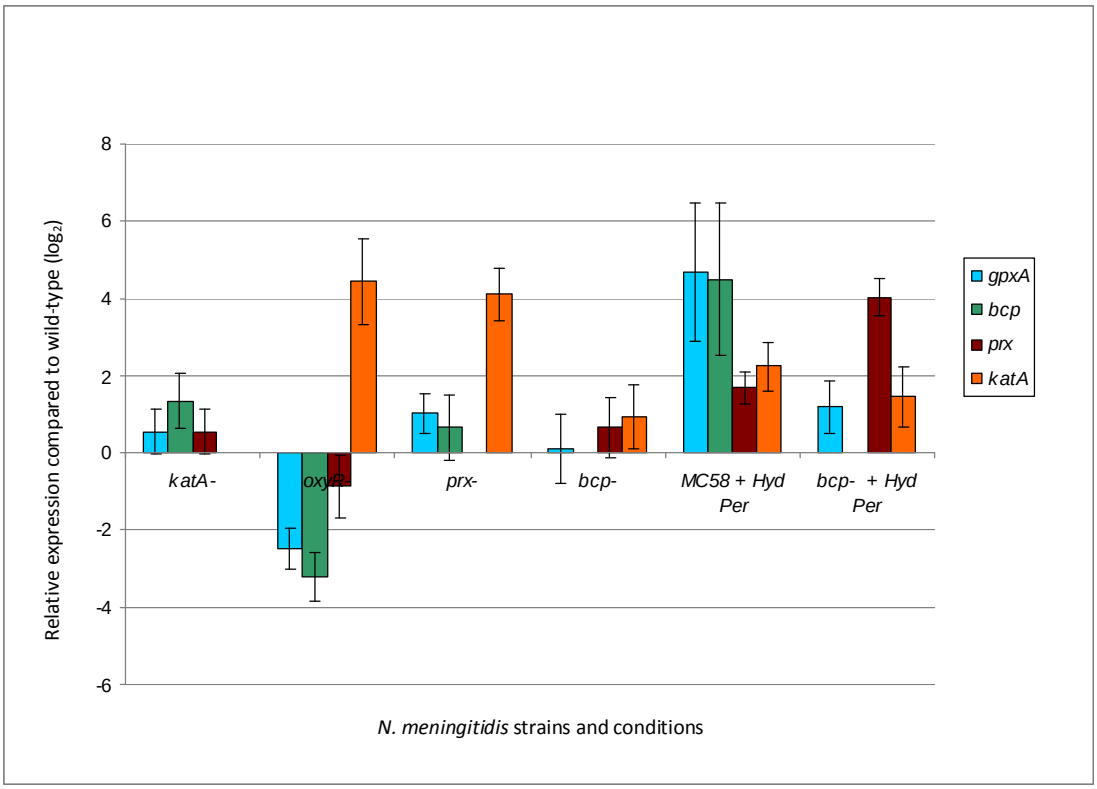


Figure 5.16. Relative gene expression levels of key *N. meningitidis* peroxidsae stress response genes. Error bars represent 1 standard error. It can be seen that mutants deficient in *oxyR* displayed elevated expression of the gene *katA*, encoding catalase. *prx* mutants also showed elevated expression of *katA*.. *bcp*, *prx* and *gpxA* are all regulated in a peroxide / *oxyR* dependent manner, as expression of these genes are all significantly decreased in an *oxyR* mutant. Mutating *bcp* may increase expression of *prx* or *gpxA* but this was not statistically significant. *prx* expression was up-regulated further in a *bcp*::Ery mutant than in the wild type strain following exposure to low-level hydrogen peroxide.

As demonstrated in earlier experiments shown in this chapter, the *N. meningitidis bcp*::Ery strain survives better in co-culture with *S. pneumoniae* than does the wild-type MC58 strain (see Figure 5.8). The absence of *bcp* leads to a minor increase in expression of *prx* and *katA* under standard culturing conditions (see Figure 5.16). Given that *prx* deficiency decreases survival of *N. meningitidis* in co-culture, we reasoned that the elevated survival of *N. meningitidis* in the absence of *bcp* may be due to increased expression of *prx* in this strain. Indeed, *prx* expression is up-regulated further in response to hydrogen peroxide in a *bcp*-deficient mutant than in the wild type (see Figure 5.16).

To test this further we examined relative expression of the peroxidase stress response genes during co-culturing of wild-type *N. meningitidis* MC58 and the *bcp*::Ery strain with *S. pneumoniae*, by RT-PCR. The results of these experiments are shown in Figure 5.17 overleaf. The three identified peroxidases and catalase are up-regulated during the co-culture, in line with the expectation, given that the *S. pneumoniae* creates oxidative stress conditions. However, the extent of induction of *prx* is less in the *bcp* deficient strain than in the wild type. Induction of *gpxA* and *katA* is also marginally less in *bcp*::Ery, when compared to the wild-type. Apparently, in the absence of *bcp*, there is less induction of peroxide responsive genes and less oxidative damage, suggesting that an alternative pathway or pathways for preventing oxidative damage occurs in this strain under co-culture conditions.

Figure 5.17.

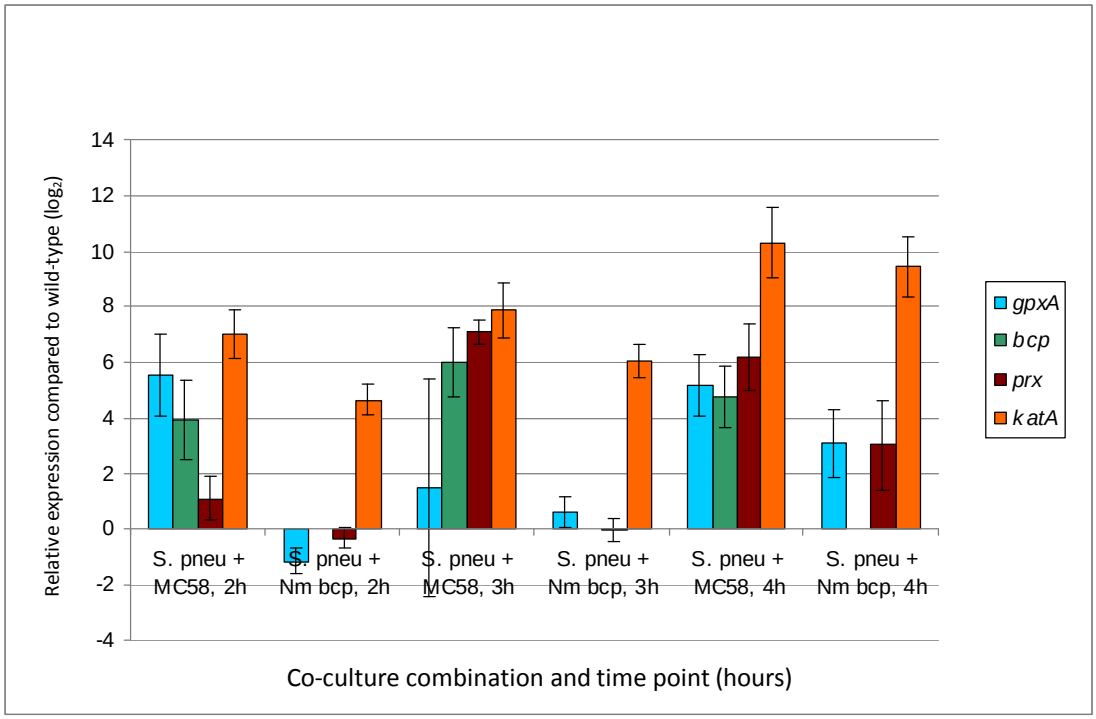


Figure 5.17. Relative gene expression levels of *N. meningitidis* key peroxide stress response genes in liquid co-culture experiments. Error bars represent 1 standard error. The three identified peroxidases and catalase were upregulated during the co-culture, in line with the expectation. However, the extent of induction of *prx* wass less in the *bcp*-deficient strain than in the wild-type. Induction of *gpxA* and *katA* was also marginally less in *bcp*::Ery, when compared to the wild-type.

5.3 Discussion.

5.3.1 Liquid co-culture experiments identify two mutant strains whose behaviour deviates from that of wild-type *N. meningitidis* when in co-culture with *S. pneumoniae*.

The liquid co-culture experiments performed in this chapter were designed to investigate the importance of key peroxide stress response genes in *N. meningitidis*, when the strains were exposed to peroxide stress from *S. pneumoniae*. The original hypotheses formed when designing these experiments support *katA* being crucial for survival of *N. meningitidis* in co-culture with *S. pneumoniae*, but the data do not support this. Rather, Prx seems to be important since *prx* mutants survive particularly poorly in the co-culture experiments.

The liquid co-culture experiments showed the *prx::*Spec mutant strain to be more sensitive to hydrogen peroxide than wild-type *N. meningitidis* MC58. This is very different to the observations of Seib et al. (2007), when analysing *N. gonorrhoeae*. This study showed the expression of *prx* in N. *gonorrhoeae* to be increased 6-fold in the presence of 1mM hydrogen peroxide, with a *prx* mutant demonstrating increased resistance to hydrogen peroxide stress when compared to wild type *N. gonorrhoeae* which was due to an increased expression of *katA* (Seib et al., 2007).

The study by Seib et al. (2007) indicated that a *prx* mutant is highly resistant to high level hydrogen peroxide, whereas, the concentration of hydrogen peroxide that the bacteria are exposed to in the experiments carried out in the present study were much lower. Previous results from this study, shown in Chapter 4, indicate a hydrogen peroxide concentration of around 75 µM being produced in a liquid co-culture containing both *S. pneumoniae* and *N. meningitidis* MC58. This

concentration is 13.3-fold lower than that used by Seib et al. (2007) and could explain the difference in results observed, as this concentration of hydrogen peroxide may not be high enough to induce an OxyR-mediated response to the peroxide toxicity, whereas, 1mM hydrogen peroxide used previously (Seib et al., 2007) is a sufficient concentration to activate OxyR and cause the de-repression of the *katA* gene (Ieva et al., 2008), leading to high catalase production and an increased resistance to the hydrogen peroxide toxicity.

A further interesting result from the co-culture experiments involving mutant *N. meningitidis* strains was observed from co-cultures containing *S. pneumoniae* and the *bcp::*Ery mutant strain. During these co-culture experiments, the lack of the *bcp* gene, NMB0750, promoted survival of the mutant strain when in co-culture. As a lack of the *bcp* gene promotes growth and survival of *Neisseria* in co-culture with *S. pneumoniae*, it suggests that an alternative gene, or genes, is / are upregulated to compensate for the lack of the Bcp protein being expressed. This upregulation may offer protection against the toxic peroxide, allowing the *bcp::*Ery mutant to survive and grow when in co-culture with *S. pneumoniae*.

5.3.2 Building a model of the hydrogen peroxide response pathway in *N. meningitidis*.

In order to reason this hypothesis further, a model of the hydrogen peroxide response in *N. meningitidis*, along with how the *bcp* gene is involved in this response was formed;

The *bcp::*Ery mutant strain of *N. meningitidis* was able to survive when exposed to peroxide stress created by *S. pneumoniae*. To aid this increased survival, the expression of an alternative gene was upregulated, to compensate for the lack of the *bcp* gene. A candidate gene is *prx*, as it

is involved in the peroxide response pathway of *N. meningitidis*, via the *oxyR* regulon (Seib et al., 2007). The function of both Prx and Bcp is to scavenge low-level peroxides in cells (including organic peroxides), which keeps cellular concentrations of peroxides low. Thus, if there is a functional Prx or Bcp present in the cell, the concentration of hydrogen peroxide is prevented from rising to a level where *katA* is expressed in an OxyR-dependent manner. If katA were not up-regulated under low/intermediate concentrations of hydrogen peroxide, this could explain why a *katA* deficient strain of *N. meningitidis* behaves no differently to the wild type MC58 strain in co-culture. However, the results presented here show that *katA* is up-regulated under conditions relevant to the hydrogen peroxide concentration present during co-culture with *S. pneumoniae*. Therefore, it appears more likely that the lack of protective effect from *N. meningitidis* catalase arises either because it does not have a sufficiently high peroxide affinity to effectively remove hydrogen peroxide at low concentrations, or that the long-term cellular damage in co-cultures is due to organic peroxides, which catalase does not detoxify.

The results obtained from co-culture experiments carried out in this chapter can be explained in terms of this model. Differences in *N. meningitidis* mutant strain survival, both positive and negative when compared to the wild type MC58 strain, were only observed when the low-level peroxide scavenger peroxidases were deleted (i.e. in *bcp*::Ery and *prx*::Spec deletion strains). This indicates that the strains in the co-cultures were exposed to low / intermediate hydrogen peroxide concentrations and that this concentration was not high enough to be affected by catalase expression. Indeed, as shown in Chapter 4, hydrogen peroxide concentrations in co-cultures were low (~ 75 µM), whereas catalase conferred a protective effect only at higher concentrations of hydrogen peroxide (such as 1 mM) (Seib et al., 2007).

Further to this, it is interesting to build a model of how the hydrogen peroxide level is controlled, allowing a *bcp*::Ery mutant to survive when in co-culture with *S. pneumoniae*; a lack of the *bcp* gene leads to increased

intracellular hydrogen peroxide concentrations, due to the lack of low-level peroxide scavenging by Bcp. The concentration of hydrogen peroxide rises to an intermediate level, where *prx* is activated by OxyR and is therefore up-regulated in terms of its expression and activity. It is also possible that *prx* is expressed at low-levels but is simply up-regulated further due to the increased hydrogen peroxide concentration. The up-regulation and increased activity of *prx* leads to hydrogen peroxide breakdown, causing the intracellular concentration to fall, leading to downregulation of *prx*. This decrease in expression and activity of *prx* then causes hydrogen peroxide concentrations to rise once again, as there is no Bcp present to scavenge the low-level peroxide, hence the cycle continues to repeat.

5.3.3 RT-PCR experiments give insight into the expression levels of key genes of the eroxide stress response pathway and suggest that *N. meningitidis* has multiple mechanisms for dealing with peroxide stress.

In addition to building a model of the peroxide stress response pathway, it was important to assess the expression levels of all key genes involved, hence RT-PCR experiments were performed in order to assess the impact of genotype and environment on the transcription of several peroxide stress response genes.

These RT-PCR experiments showed that mutants deficient in *oxyR* displayed elevated expression of the gene *katA* following culture of *N. meningitidis* in the absence of any exogenous oxidative stress (Fig. 5.16). This is in agreement with previous observations in *N. gonorrhoeae* (Tseng et al., 2003) and *N. meningitidis* (Ieva et al., 2008), that OxyR is a repressor of *katA* expression in the absence of peroxide. Furthermore, *prx*::Spec mutants also showed elevated expression of *katA*. This may be

due to *prx::Spec* mutant strains being under increased levels of oxidative stress as they cannot break down low / intermediate concentrations of hydrogen peroxide, which causes the peroxide concentration to rise to high levels, leading to OxyR-dependent de-repression of *katA* in accordance with the model described in this discussion. This is in keeping with the observation made by Seib et al. (2007) that, in *N. gonorrhoeae*, catalase is upregulated in *prx* mutants.

Furthermore, the *bcp*, *prx* and *gpxA* genes are all regulated in a peroxide / oxyR dependent manner. RT-PCR experiments carried out in this chapter show that the expression of *bcp*, *prx* and *gpxA* were all significantly decreased in an *oxyR*::Tet mutant (Figure 5.16), indicating that this regulator is an activator of peroxidase gene expression. Microarray studies with *N. gonorrhoeae* previously identified *prx* as part of the *oxyR* regulon (Seib et al., 2007), but not *bcp*; *gpxA* is absent from *N. gonorrhoeae* (Moore and Sparling, 1995). Mutating *prx* caused elevation of the expression of *bcp* and *gpxA*, giving further support to the notion that the *prx*::Spec mutant strain is under oxidative stress. Mutating *bcp* may increase expression of *prx* or *gpxA* but this was marginal. This is interesting to note, as this does not support the hypothesis that *prx*, or another peroxidase, is up-regulated in a *bcp*::Ery mutant, leading to its ability to survive when in co-culture with *S. pneumoniae*. Indeed, it appears that *prx* is more important in the peroxide stress response, as there is definite increased peroxidase expression in a *prx*::Spec mutant.

The data from these RT-PCR experiments are consistent with the model described in this chapter. It appears that *N. meningitidis* utilises a stress response system in which several peroxide resistance mechanisms are expressed in order to deal with oxidative stress, in which catalase is required for dealing with high peroxide concentrations. Endogenous *N. meningitidis* catalase, however, does not equip the bacterium to deal with stresses caused by continuous low level peroxide production, such as is found during co-culturing with a hydrogen peroxide synthesising bacterium like *S. pneumoniae*. The sensitivity of *prx*::Spec mutants during the co-

culture experiments indicates that the *prx* gene product is primarily responsible for conferring resistance in the wild-type *N. meningitidis*.

5.3.4 Increased resistance of the bcp::Ery strain of *N. meningitidis* to killing by *S. pneumoniae* in co-culture experiments indicates that alternative peroxide resistance systems may be available to *N. meningitidis*.

Initial liquid co-culture experiments carried out in this chapter show that the *N. meningitidis bcp*::Ery strain survives better in co-culture with *S. pneumoniae* than does the wild-type (Fig. 5.8). The absence of the *bcp* gene leads to a minor increase in expression of *prx* and *katA* under standard culturing conditions, as shown by RT-PCR experiments in Figure 5.16. As discussed previously in the model of regulation proposed here, it was reasoned that the elevated survival of *N. meningitidis* in the absence of *bcp* may be due to increased expression of *prx* in this *bcp*::Ery mutant strain. In order to test this hypothesis, RT-PCR experiments were carried out on samples from liquid co-culture experiments between *S. pneumoniae* and *N. meningitidis* wild-type MC58 and *bcp*::Ery mutant strains, to examine the relative expression of the peroxidase genes during co-culture (See Figure 5.17).

These RT-PCR experiments showed that the three identified peroxidases (Prx, Bcp and GpxA) and catalase were upregulated during co-culture experiments with S*. pneumoniae*. This is in line with expectations, given that the *S. pneumoniae* creates oxidative stress conditions. However, the extent of induction of *prx* expression was less in the *bcp*::Ery strain than in the wild-type MC58 strain. Induction of *gpxA* and *katA* expression was also marginally less in a *bcp*::Ery mutant, compared to the wild-type. Apparently, in the absence of *bcp*, there is less induction of peroxide responsive genes and less oxidative damage, suggesting that an alternative pathway or pathways for preventing oxidative damage occurs in this strain under co-culture conditions.

It is also worth noting that the survival of a *bcp*::Ery.*prx*::Spec double mutant of *N. meningitidis* in co-culture with *S. pneumoniae* is very poor

(Fig. 5.12). Thus, the enhanced survival of the *bcp*::Ery mutant strain in co-culture is not retained in the absence of *prx*, indicating that the *prx* gene is dominant over *bcp* and suggesting that the enhanced survival capacity of a *bcp*::Ery mutant may be related to *prx* function, although this may be a regulatory effect at a post-transcriptional level as there is little increase on *prx* expression is seen in a *bcp*::Ery mutant either in single species culture, or in co-culture with *S. pneumoniae*. This is in agreement with the model described in this chapter and is consistent with the previous results from RT-PCR experiments discussed here, although it appears that the involvement of *prx* may not be at the gene expression level as first predicted.

It is, however, possible that the *bcp*::Ery strain of *N. meningitidis* performs better than wild type MC58 strain when in co-culture with *S. pneumoniae*, simply because it has slightly up-regulated *prx* and *katA* gene expression, even under un-stressed conditions (See Figure 5.16). This may mean that the *bcp*::Ery strain is able to deal with the transition to oxidative stress in the presence of *S. pneumoniae* better than other mutant strains tested in these experiments.

5.3.5 OxyR-dependent regualtion of catalase production protects *N. meningitidis* from toxic bursts of hydrogen peroxide.

The various *N. meningitidis* strains were exposed to a high concentration burst of hydrogen peroxide, to investigate whether their behaviour was consistent, or differed to that observed when the strains were in co-culture with *S. pneumoniae* (and therefore exposed to low-level hydrogen peroxide). In contrast to co-culture results, both *oxyR*::Tet and *prx*::Spec mutant strains were highly resistant to the burst of hydrogen peroxide (See Figure 5.14).

The up-regulation of catalase production observed in *oxyR*::Tet and *prx*::Spec mutant strains from RT-PCR experiments (See Figure 5.16), serves as a reasonable explanation for the resistance of the *oxyR*::Tet and *prx*::Spec mutant strains to a burst of hydrogen peroxide. The wild-type MC58 strain and the *katA*::Kan mutant strain do not express catalase following growth under standard non-stressful conditions. But, it has been shown previously that catalase is required to deal with high concentrations of peroxide (Seib et al., 2007), so *oxyR*::Tet and *prx*::Spec mutant strains survive effectively in the hydrogen peroxide burst assay compared to wild-type MC58 and *katA*::Kan mutant strains, due to constitutive expression of catalase that is oxyR-dependent (See Figure 5.13).

If this hypothesis is correct, it would be expected that pre-treatment of wild-type *N. meningitidis* cells with a non-toxic dose of hydrogen peroxide sufficient to induce catalase gene expression to protect the culture against high-level, 1 mM hydrogen peroxide. This is indeed the case as is shown in Figure 5.14. It has been shown here that pre-treatment with 20 µM hydrogen peroxide peroxide is sufficient to induce catalase and protect *N. meningitidis* from a burst of 1 mM peroxide. The *N. meningitidis katA*::Kan mutant strain was not protected from killing by 1 mM peroxide following pre-treatment with 20 µM hydrogen peroxide (See Figure 5.14).

5.3.6 Concluding remarks.

The results in this chapter have given an insight into the effect of key genes in the *N. meningitidis* response pathway and have identified that Nm *bcp* and Nm *prx* are both important genes in the hydrogen peroxide stress response pathway. After building a stress response model for *N. meningitidis*, it was hypothesised that the ability of the *bcp*::Ery strain to survive well in co-culture with *S. pneumoniae* may be as a result of the upregulation of the Nm *prx* gene. In fact, it was shown that there are probably multiple mechanisms acting in this pathway and that Nm *prx* is important for protection of *N. meningitidis* against toxic peroxides. It was also shown that it may not be as easy as first thought to explain the results observed with the *bcp*::Ery strain.

It is, at present, not known whether the involvement of *prx* in the *bcp*::Ery mutant strain's ability to survive against *S. pneumoniae* is at the transcriptional or post-transcriptional level, although it appears that the function of *prx* is important, as well as its expression levels. Further experiments are needed in order to investigate this. As discussed, it is also possible that the increased survival of the *bcp*::Ery strain is simply down to slightly increased *prx* and *gpxA* expression seen in this strain under non-stressed conditions.

6. Further work.

This study has acheived the aims set out in chapter 1 and has given some valuable insights into the microbial diversity of the human mouth, revealing many genera from each bacterial phyla and showing *Streptococcus*, *Neisseria* and *Veillonella* to be the prevalent genera, as idenfied via RFLP and FAME profiling analyses. It would be interesting to re-visit this diversity analysis, as a separate study, in order to analyse many more samples and obtain a data set that is more statistically sound, as well as gaining more of an insight into the stability of the oral microflora and the changes that take place over time. It would also be interesting to investigate whether, given further time and attention to the diversity analyses, an increased number of genera would be identified, or whether the same number and types of genera would be identified in higher amounts. Given the small scale of the diversity study carried out in this work, it is reasonable to presume that a larger diversity study would reveal an increased number of genera, although it must be presumed that the genera already identified would also be seen in future analyses.

As it was not possible to detect food-borne microbes in the mouth following a salad meal, it would be interesting and valuable to invest some further time into repeating these time-course studies, analysing many more clones over more time points during the experiment. This would enable firmer conclusions to be drawn from the data, as there would, again, be a larger data set that statistics could be performed on. It would also be interesting to use different methodologies to detect the bacteria, such as RT-PCR and high-throughput 454-DNA sequencing, that may be more powerful and sensitive, detecting the microbes from smaller amounts of DNA that may be present as the microbes are washed from the mouth. It would be interesting to investigate whether repeating these experiments as described above would result in the same observation, that food-borne microbes cannot be detected in the mouth after eating.

The interactions between the dominant genera identified from the oral cavity were investigated by performing bacterial overlay experiments. It would be interesting to repeat these overlay experiments using a combination of an oral and a food-borne microbe. This would give an insight into whether there are detectable interactions between such microbes, even though the presence of food-borne microbes cannot be identified in the oral cavity at this time. It would be interesting to learn whether oral microbes are capable of killing food-borne microbes, which may help to reason the absence of food-borne microbes in the mouth during and immediately after eating.

Several oxidative response genes of *N. meningitidis* were investigated, following the observation that *Neisseria* was killed when in co-culture with *Streptococcus* and the mediator of this hypothesised to by hydrogen peroxide. Given an extended period of time, it would be interesting to perform a microarray analysis to investigate changes in gene expression levels on a much wider scale and allow the analysis of many more genes. Previous microarray studies to investigate the oxidative stress response were performed in *N. gonorrheae* and it would be interesting to compare results of such a study being performed in *N. meningitidis*. It is reasonable to presume that the number of genes whose regulation is affected by oxidative stress is much larger than the small number analysed in this study and the results of a large microarray study may offer a greater insight into the oxidative stress response of *N. meningitidis*.

Abbreviations.

RFLP	Restriction Fragment Length Polymorphism
T-RFLP	Terminal Restriction Fragment Length Polymorphism
FAME	Fatty Acid Methyl Ester
RT-PCR	Real -Time Polymerase Chain Reaction
MIDI	Microbial Identification System
ROS	Reactive Oxygen Species
NOX	NADPH Oxidase
AhpC	Alkylhydroperoxidase
SOD	Superoxide Dismutase
PBS	Phosphate Buffered Saline
HBA	Horse Blood Agar
BHIBLA	Brain-Heart Infusion Agar, supplemented with Horse Blood
NA	Nutrient Agar
MHB	Mueller Hinton Broth
TSB	Transforming Sotrage Buffer
CTAB	Cetyltrimethylammonium Bromide
SDS	Sodium Dodecyl Sulfate

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